University of Alberta

Effects of intestinal surgery on physiological determinants of Crohn's disease

By

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ABSTRACT

Crohn's disease often requires surgery, but resection of involved bowel is usually followed by recurrence at the anastomotic site. It is possible that the response to intestinal surgery could stimulate disease recurrence, and that this response might be most pronounced in animals predisposed to inflammatory bowel disease.

Intestinal permeability and inflammation was measured following intestinal transection/reanastomosis in wild type and interleukin-10 knockout mice, and compared with results from animals that had undergone sham surgery.

Following surgery, wild type mice developed histological inflammation within millimeters of the incision. Their small intestinal permeability was globally decreased. Both pro and anti-inflammatory cytokines peaked within three centimeters of the incision. One week postoperatively, their splenic lymphocytes were hyporesponsive to stimulation, suggesting a normal state of immune downregulation. IL-10 deficient mice had wider histologic insults and a loss of immune downregulation. Their postoperative permeability changes were normal.

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LIST OF ABBREVIATIONS

5-ASA	5-aminosalicylate
6-MP	6-mercaptopurine
AZA	azathioprine
BSA	bovine serum albumin
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
FCS	fetal calf serum
IFN-γ	interferon gamma
IL-1β	interleukin 1 beta
IL-6	interleukin 6
IL-8	interleukin 8
IL-10	interleukin 10
IL-10KO	interleukin 10 knockout
IL-16	interleukin 16
IPAA	ileal pouch-anal anastomosis
LPS	lipopolysaccharide
PCR	polymerase chain reaction
PD	potential difference
TGF-α	transforming growth factor alpha
TGF-β	transforming growth factor beta
TMB	3,3',5,5'-tetramethylbenzidine

TNF-α	tumor necrosis factor alpha
WT	Wild-type (Sv/Ev129)

CHAPTER 1

INTRODUCTION

BACKGROUND

Crohn's disease is a chronic inflammatory bowel disease that has challenged clinicians for decades because of its variability of patient presentation, complex pathophysiology and as yet incurable nature. It has a widely variable geographic prevalence, which rises at increasing distances from the equator, and an incidence that is increasing with time(1). Central Canada, an area of relatively high Crohn's prevalence, has reported a population prevalence of approximately two per 1000 population (2), with data from individual centers reaching prevalence of five per 1000. Although, as a population, patients with Crohn's disease as do not have increased mortality, Crohn's does result in considerable morbidity, as well as direct and indirect health care expenditure(3, 4).

Perhaps the most vexing clinical setting in Crohn's disease is that of the postoperative patient. When followed up long term, more than three quarters of patients with Crohn's disease will require surgery, and a high proportion of those patients will require reoperation(5). Despite new treatment advances and the fact that such a large proportion of patients require surgery, evidence is lacking that medical therapy can increase the interval until reoperation is necessary.

A version of this chapter has been published. Penner *et al* 2005. *Inflammatory Bowel Diseases; 11(8) : 765.*

Surgery is often necessary for complications of Crohn's disease, and intestinal resection has resulted in satisfactory outcomes for many patients(6) but the necessity for multiple operations can risk iatrogenic short-bowel syndrome and may result in progressively more challenging procedures as a result of surgical adhesions. These factors make the study of postoperative Crohn's disease etiology and management central to our understanding of the disease process.

Unfortunately, research in this area has been limited by certain challenges. Human studies have been heterogeneous due to the lack of a universally applied manner for defining recurrence. Histologic, endoscopic and clinical criteria have all been used in this setting, but their correlation is imperfect(5, 7-9), and standardized clinical assessment tools for measuring Crohn's disease activity are not necessarily useful in the postoperative setting(8). Long term follow-up of postoperative recurrence may be further complicated by changes over time as a result of improving surgical technique and the evolving epidemiology of inflammatory bowel disease. Finally, results from different centers may vary as a result of patient demographic factors and local perioperative care patterns. The use of animal models to study disease mechanisms becomes, by necessity, more complicated and labor-intensive if a relevant postoperative setting is to be studied.

Current management of postoperative Crohn's disease is an unfortunate story. The tale is one of therapies being used blindly, such as antibiotics without a specific bacterial target. Other treatments are immunosuppressant, but they are applied

without knowledge of how surgery affects the immune system, simply because they have a good track record in other clinical settings. Finally, there has been no means of predicting which patients are likely to relapse following surgery, and when their disease might recur. Thus, it is probable that postoperative medical therapy for Crohn's disease is sadly inadequate for some, and unnecessary for others. Will the story have a happy ending? The answer depends on the success of further research.

In this chapter, an attempt will be made to synthesize the available data on postoperative Crohn's disease prognosis, pathophysiology of disease recurrence in this setting, and current therapies to reduce postoperative disease burden. In conclusion, attention will be paid to areas requiring further research, and their importance in improving available patient care.

SURGERY FOR CROHN'S DISEASE

Surgery for Crohn's disease may be necessary for specific surgical emergencies, such as perforation or intestinal obstruction. It may also be used for the control of symptoms when medical therapy is not useful, such as in the case of fibrotic strictures, or in the setting of inflammatory disease that is refractory to medical therapy. While Crohn's disease can contribute to surgical necessity for a variety of other reasons, including abscess drainage, perianal fistulas, and even hip replacement, the review in this chapter will be limited to a discussion of surgery used for the management of intestinal disease.

INCIDENCE OF SURGERY

Studies that have followed Crohn's patients from diagnosis have found variable rates for the requirement of intestinal surgery but the rates are consistently high. Of 615 patients followed at the Cleveland Clinic Foundation for a mean of 13 years, 438 required operation(10), and the National Cooperative Crohn's Disease Study of 569 patients found a cumulative probability of surgery of 78% at 20 years from symptom onset(5). Given that surgery might be required at any site of disease, it is not surprising that incidence of first resection appears to be highest in patients with ileocolonic disease, where the Cleveland Clinic group demonstrated a cumulative probability of resection of 91.5% after a mean of 13 years(11). In their series, 66% of patients with Crohn's limited to the small intestine required surgery as compared with 58% of patients with only colonic disease(11). A similar pattern was seen in the National Cooperative Crohn's Disease Study(5).

INDICATION FOR SURGERY

Surgical indications have varied widely between retrospective studies assessing long-term follow-up of Crohn's patients, but the most common reasons for resection have traditionally been intestinal obstruction or medical intractability. In large studies, failure of medical therapy has been cited as the reason for resection in anywhere from 16 to 68% of patients, and obstruction in 22 to 45%(5, 6, 12). The management of

fistulas influences surgical indication in approximately 10-25% of patients(5, 6, 12, 13). Many of the patients in these large studies had surgery in the 1970's and 1980's, and whether the advent of newer treatment modalities in the 1990's will have a large effect on surgical indications remains to be seen. Strictureplasty, a bowel-conserving surgical technique used specifically for the management of intestinal strictures will be discussed in more detail in a later section. Its indication is predominantly for intestinal obstruction(14).

POSTOPERATIVE RECURRENCE

Following resection, recurrence of Crohn's disease is almost inevitable when patients are followed up long-term, and usually occurs at the site of surgical anastomosis(15). As a population, Crohn's patients predictably develop endoscopic recurrence and later develop clinical symptoms. Rutgeerts and colleagues found endoscopic evidence of Crohn's recurrence in 73% of patients one year after surgery, but only 20% of patients had any symptoms(16). When they repeated their observations at three years postoperatively the endoscopic recurrence rate had increased to 85%, and symptoms were present in 34%. Endoscopic recurrence was demonstrably accompanied by classic histological changes of Crohn's disease in areas that had been normal following surgery(16). Subtle aphthous ulceration is typically followed by serpiginous ulceration and thickening of small intestinal folds(15).

Strangely, the smooth graphs that can be drawn to represent these different measures of recurrence are of little use to individual Crohn's patients, since the development of one form of recurrence correlates poorly with other measures. Specifically, endoscopic lesions develop without necessarily producing symptoms(9, 15), histological changes do not correlate with the likelihood of clinical recurrence(7), and early endoscopic recurrence does not correlate with later surgical findings(17).

Perhaps the most relevant clinical outcome following intestinal resection for Crohn's disease is the requirement for a second surgery. When assessed in large studies, rates of reoperation have mirrored the incidence of first surgery, in that ileocolic disease has been generally associated with higher rates of reoperation than isolated ileal or colonic disease(5, 10). Smaller individual studies, however, have occasionally shown no difference in reoperation rate(13, 18) or even a higher rate of reoperation in patients with isolated ileal disease(19). In the National Cooperative Crohn's Disease Study, over 70% of patients who underwent resection for ileocolitis required a second surgery within 15 years, and the median time to reoperation was between 5 and 10 years. Similarly, after a median of 13 years of follow-up, Whelan *et al* demonstrated reoperation rates of 53% for ileocolonic disease, 45% for colonic disease, and 44% for small intestinal disease. While results vary from center to center, the trend that approximately half of patients undergoing ileocolonic resection will require reoperation within about 10 years has been fairly consistent between studies(6, 12, 20).

Many studies have attempted to define predictors of recurrence. The largest of these was a retrospective analysis of 1379 patients by Michelassi *et al*, which identified only the number of intestinal sites involved as an independent factor associated with reoperation(12). That a greater extent of disease preoperatively is associated with a higher risk of recurrence has been verified in other studies where recurrence is defined in terms of symptoms(21-23) or as reoperation(24). It has also been verified in the pediatric age group(25).

The search for predictors of recurrence includes one study showing a decreased recurrence rate in smokers who quit following their resection as compared with those who continued smoking(26). One retrospective study also found that smoking more than doubles the risk of symptomatic or surgical recurrence(27) but a large prospective trial failed to show that smoking is independently associated with a high relapse rate(28). The presence of preoperative granulomas has been suggested as a predictor of recurrence but this has been controversial(29, 30). Preliminary findings have suggested that the occurrence of postoperative complications may predict a higher recurrence rate(31) and patients with perianal disease may be prone to early recurrence(32).

Based on data suggesting a bimodal distribution of postoperative Crohn's recurrence, some authors have suggested that there may be a subgroup of Crohn's patients at particular risk of reoperation and that these patients may be more likely to present as intestinal perforation(31, 33-35). A study of 280 patients designed to test the

hypothesis that distinct Crohn's subgroups exist did not supported that theory(19). Another retrospective study by Yamamoto *et al*, suggested that perforating disease does not carry an increased risk of recurrence, but when it does recur, is more likely to present with a second perforation(36). Patients presenting with intestinal perforation fortunately represent a small minority of Crohn's cases, and the question of whether they have an increased relapse rate remains controversial. Given that when they do return with recurrence it is more likely to be catastrophic, many clinicians consider them a high-risk group, while others question the validity of classifying patients into risk groups with the current evidence.

SURGICAL TECHNIQUE

Increasing surgical experience and research in the management in Crohn's disease has led to an evolution in favor of a more conservative approach. Traditionally, as with cancer, it was intuitive that the complete resection of all involved bowel would be more likely to result in a 'curative' resection. A wide resection margin surrounding normal tissue was often taken, and some surgeons even obtained frozen sections to ensure that all microscopic disease had been removed. The high recurrence rate that was documented even after extensive resection(5) suggested that this technique was ineffective, and further research demonstrated that recurrence rate was unaffected by the presence of microscopic disease at surgical margins(37, 38) or by increased length of resection(39). The application of a "bowel-sparing policy", in which only grossly diseased tissue was resected was found to be reasonable in Crohn's colitis as well as

ileitis, and stood up to prospective randomized trial designs following initial retrospective analysis(40, 41).

Another clinical setting in which an increasingly conservative approach has become popular in the management of Crohn's disease is in the treatment of small intestinal strictures. Traditional resection of strictures has remained a viable option, but in patients with multiple strictures it can result in short-bowel syndrome. Several techniques, referred to as strictureplasties, have been put into practice by which the intestine is incised and its lumen widened without resection. Application of these techniques in multiple centers has demonstrated that they are safe and effective, and that the recurrence rate of Crohn's disease is not increased as compared with resection(42-45). The various surgical techniques for strictureplasty have all had technically good results and the choice of technique will generally depend on the stricture length(46, 47), but one meta-analysis of 1825 strictureplasties suggested that reoperation rate and Crohn's recurrence rate was lower for the Finney technique than the Heinecke-Mikulicz(14). Strictureplasties can be used for isolated strictures but also appear to be reasonable for diffuse jejuno-ileal disease(48), and have been used with good results for long strictures in the pediatric population(49). Technically, it has been noted that multiple strictureplasties within close proximity of one another can result in a relatively immobile segment of bowel with high tension on suture lines(46). Some authors have cautioned that strictureplasty is associated with a high rate of postoperative complications when used for duodenal Crohn's disease, but this has not been observed in all centers(50, 51).

Other surgical techniques have been developed in attempts to improve postoperative prognosis. Terminal ileal resection will often involve loss of the ileocecal valve and consequent reflux of colonic bacteria into the small bowel. This has been proposed as a mechanism perpetuating or inducing recurrence of the neoterminal ileum, so attempts have been made to develop surgical techniques that preserve a one-way valve effect. Two observational studies have assessed patients following the formation of a nipple-valve ileal anastomosis, and postoperative recurrence rates have been low as compared with historical experience(52, 53). As yet, though, no studies have directly compared this method with traditional ileostomy techniques.

Further research has concentrated on specific anastomosis techniques, and how they might alter the risk of Crohn's recurrence. Concern has been raised that anastomotic stapling techniques result in a localized ischemic injury that might increase Crohn's recurrence(23, 54). Conversely, suture materials have been found to alter macrophage function and this has been proposed as a reason why hand-sewn anastomoses might be at higher risk(55). Clinical studies have had variable results. Some retrospective studies have found lower recurrence rates in stapled than hand-sewn anastomoses(56, 57), while others have found no difference(27, 58). One non-randomized prospective study found a lower rate of recurrence in the patients with stapled anastomoses(59). Firm conclusions are difficult to draw at this time because surgical sites, specific techniques and follow-up times vary between studies, and no randomized trials exist. It can be said, however, that no evidence currently supports

the hypothesis that stapled anastomoses result in a higher recurrence rate than hand-sewn.

POSTOPERATIVE COMPLICATIONS

Modern bowel-sparing surgical procedures can typically be performed with low postoperative morbidity and a recent meta-analysis of 1825 strictureplasties found zero mortality(14). Rates of postoperative complications vary widely between studies, largely based on which end points are considered complications, but rates of specific complications such as leaks are fairly consistent. A retrospective analysis of 1124 strictureplasties published in 2001 found that patients had a median hospital stay of eight days, postoperative mortality of zero percent, and overall postoperative morbidity of 18%. In this case, the relatively high morbidity is due to a very inclusive definition. Of the 18%, 5% were septic complications, of which two percent were leaks. Only four patients (just over 1%) required an early reoperation, and a large number of the complications were less severe. 7% were made up of postoperative hemorrhage, most of which were self-limited, and 4% were counted due to prolonged ileus.

A possible contrast with the previous study by Dietz and colleagues is another retrospective analysis by Sampietro *et al*, which included patients who underwent strictureplasty or conservative resection. Again, they found zero postoperative mortality, but they reported only 5.8% morbidity(60). On closer inspection, they

report that half of those patients (2.9%) required reoperation for septic complications; this number is more in keeping with Dietz' study. A third retrospective analysis of strictureplasties reports no postoperative mortality and an overall postoperative morbidity of 12%, intermediate between the two previous studies(46). Once again, their rate of postoperative leak was about 2%. Other complications, such as prolonged ileus in about 4%, also mirror the previously stated results.

Less data is available regarding colonic surgery but one study described 103 segmental colonic resections performed with zero mortality and overall 14% morbidity(41). They had a higher rate of serious postoperative complications, with leaks in 7% and obstruction in 2%.

Although a direct comparison with traditional surgical technique is not possible because of other developments in perioperative care and medical therapy, postoperative results do seem to have improved over time. A retrospective analysis of 42 patients who underwent traditional resection at the Mayo clinic between 1935 and 1982 noted that 3 patients (7%) died of their disease, one of which was in the immediate postoperative period(13). They had a total of 7 postoperative complications, which included two wound infections, one abscess, one fistula, one obstruction and one persistent perineal sinus.

An analysis of traditional resections performed more recently, in 353 patients between 1969 and 1986, had a postoperative mortality of 1.4%(21). Their

postoperative morbidity was similar to current techniques, with abscesses in 4%, leakage in 2%, dehiscence in 1% and bleeding in 1%. That the improvements in postoperative morbidity seen over time is not clearly associated with surgical technique changes is also illustrated by a more recent study of 181 patients who had resections between 1970 and 1993, in which there was no mortality, no leaks, and only a single dehiscence(6). Most of their postoperative morbidity was accounted for by prolonged ileus in 7.5%, pneumonia/atalectasis in 8.6%, wound infections in 6.3% and abscesses in 4%.

In summary, strictureplasties and bowel-conservative resections can be performed with negligible mortality, leaks or dehiscence in about 2% of patients, and other complications that could require reoperation in 1-5%. These results are similar to those for wider resections performed with modern techniques, and would seem to be an improvement from traditional results, in which postoperative mortality was encountered at a small but significant rate.

PATHOPHYSIOLOGY OF POSTOPERATIVE CROHN'S

PATHOPHYSIOLOGY OF CROHN'S DISEASE

A unifying disease model for inflammatory bowel disease remains elusive, but the most useful disease model currently views Crohn's as an aberrant interaction between the host immune system and resident enteric microflora. Research to date has failed

to identify a single infectious agent responsible for the disease, but it is clear from animal models that the fecal flora is mandatory for inflammation. Surgical bypass procedures further confirm the necessity of fecal flow for the expression of Crohn's. The picture is further complicated by the fact that both environmental and genetic factors must collaborate for Crohn's to occur.

Without a single causative factor to blame for Crohn's, we have come to see the disease phenotype as dependent on balancing acts. First, there seems to be a balance of relatively pathogenic and relatively protective bacteria in the bowel. Second, the bowel has a delicate balance between proinflammatory and immunosuppressant cytokines that maintains homeostasis, while still vigilant for infections. Not only the intensity, but the nature of the inflammatory response must be balanced, with Th1 and Th2 responses coordinated. The description of any of these balancing acts as the single fundamental issue in Crohn's is plagued by questions of cause and effect, but what is clear is that patients expressing Crohn's disease have lost their balance in all three areas.

Key in the pathogenesis of Crohn's disease is a relative defect in the regulatory cytokines transforming growth factor beta (TGF-β) and interleukin 10 (IL-10), that have roles in maintaining tolerance towards fecal flora through regulation of mucosal T-cell activation(61). The deregulated immune system of the Crohn's patients is defective in T-cell apoptosis and produces high amounts of Th1 associated cytokines such as interferon gamma (IFN-γ)(62, 63). Other less specific proinflammatory

cytokines are also elevated, such as tumor necrosis factor alpha (TNF-α), and the interleukins: IL-1β, IL-6, IL-8 and IL-16. Growth factors also contribute to the brisk inflammatory response, including transforming growth factor alpha (TGF-α).

The bowels of patients with Crohn's disease differ in other ways from the healthy intestine, but considerations regarding etiology of the disease are fraught with questions of cause and effect. One such parameter is intestinal permeability. Patients with Crohn's disease have increased permeability, a problem that increases exposure of the enteric immune system to enteric bacteria(64). Whether this increased permeability is a cause or a product of intestinal inflammation is not known, but it is interesting that when disease is quiescent increased permeability predicts clinical relapse(65).

NORMAL POSTOPERATIVE HEALING

Intestinal surgery necessitates the activation of healing responses which have been usefully classified into four phases: hemostasis, inflammation, fibroproliferation (or scar formation) and scar remodeling(66). Progression of the healing response through these phases involves a sequential recruitment of cells, with platelets, neutrophils and macrophages dominating the early phases. Lymphocytes make up a larger fraction of the white blood cells in later stages, as fibroblasts and epithelial cells take on a greater role in scar formation and remodeling.

The inflammatory process that occurs at wound sites appears to serve several functions. Neutrophils fight infectious agents and remove debris in the earliest stages, followed by monocytes and macrophages over two to three days. Macrophages, possibly the only white blood cells absolutely required for healing(67), coordinate cellular functions through the secretion of cytokines.

Individual layers of the intestinal wall have their own healing processes. The epithelium, which constitutes the interface between the intestinal immune system and enteric microflora critical in the pathogenesis of inflammatory bowel disease, repairs itself in three steps(68). Epithelial restitution is the first step, and consists of migration of neighboring epithelial cells to fill the defect; it occurs within hours and does not involve cell division. Epithelial cell proliferation, the second step, replenishes the available pool of cells, and finally, maturation and differentiation of those cells constitutes the third and final step.

Orchestration of the series of steps required for successful surgical wound closure is carried out by a large number of cytokines, which include growth factors, interleukins, interferons and colony stimulating factors. While a complete inventory of these molecules is neither practical, nor entirely possible, a few known molecules seem to play particularly central roles.

TGF-β represents a family of peptides produced by multiple cell types involved in wound healing. It is central to wound healing because it has functions at multiple

levels including regulation of inflammation(69), inducement of epithelial cell restitution(70), and collagen production and degradation(71). In animal models, systemic treatment with TGF-β accelerates wound healing(72) and increases wound tensile strength(73), but the immunological neutralization of certain TGF-β isoforms reduces scar formation(74). TGF-β is also the mediator through which other cytokines, including IFN-γ, exert effects on healing(70, 75).

Another group of peptides believed by experts in the field to be particularly crucial in the healing response are epidermal growth factor (EGF), and transforming growth factor alpha (TGF-α), which exerts its actions through the EGF receptor(68). These molecules are particularly important because of their property of stimulating epithelial cell growth and differentiation. It appears that the interaction between TGF-β and EGF may be quite complex, with EGF stimulating epithelial proliferation and inhibiting apoptosis, and TGF-β suppressing proliferation but inducing differentiation(76, 77).

Determinants of successful healing

Important factors in the healing of an intestinal anastomosis following surgery include local factors such as adequacy of the anastomotic blood supply, healthy tissue edges, presence or absence of regional inflammation and contamination with enteric flora. Systemic factors also play a role, such as nutrition, sepsis, medications including steroids, and other immunological defects.

Some of these factors may be of particular importance following surgery for Crohn's disease. For example, adequacy of blood supply is predominantly based on the intrinsic vasculature, but can be adversely affected by tight stapling or suturing(54). The healing process may also be adversely affected by inflammatory processes present in Crohn's patients undergoing surgery. Although the success of bowel-sparing surgical techniques has demonstrated that active Crohn's disease does not preclude effective wound healing, there are several means by which Crohn's-related inflammation can challenge the healing process. Substances produced by neutrophils, such as serine proteases and free radicals, adversely affect recovery of strength in the healing intestine, a process that may be due to increased collagen degradation or impairment of collagen structural organization(78-80). The fine balance of collagen degradation, production and reorganization that takes place as part of wound healing seems in part to be regulated by the balance of TGF-β and interleukin-1 alpha(81). Their interaction is complex, in that the cytokines can have varying effects depending on whether they are acting alone, or in concert with one another. Also complex is how this interaction might be altered in a postoperative patient with Crohn's disease, since Il-1 is thought to be an important mediator of Crohn's disease(82), as compared with the attenuating effect of TGF-β.

INTERACTION OF CROHN'S DISEASE AND POSTOPERATIVE HEALING

Inflammatory and healing responses in the bowel are the products of subtle regulatory systems, which in Crohn's, are not functioning properly. A brief review of the cytokines involved in healing of surgical wounds and those involved in the mediation of inflammatory bowel disease, is adequate to demonstrate that there must be some interaction between the two processes. The system is unfortunately too complex to predict from first principles the results of that interaction. That surgical wound healing causes a visible inflammatory response associated with molecules such as IFN-γ could suggest that surgery should stimulate inflammation associated with Crohn's. On the other hand, TGF-β is crucial to the healing response, and attenuates the Th1 variety of inflammation. It is also possible that depending on disease specifics between individuals, intestinal surgery may alter the disease course in different manners for different patients.

Another parameter known to be important in Crohn's disease and affected by surgery is intestinal permeability. Intestinal permeability is increased in patients with Crohn's disease, but normalizes when a diseased segment of bowel is resected(83). While this may seem intuitive, it is somewhat surprising, since the permeability defect in Crohn's disease likely extends beyond the area of macroscopically inflamed bowel, as evidenced by the fact that permeability defects exist even when disease is quiescent(64). This raises the question of whether the intestinal surgery itself alters bowel permeability, a question that is not easily answered by the current literature. Patients who have undergone surgery for resection of cancer have demonstrated

increased permeability postoperatively(84), but patients undergoing ileal pouch-anal anastomosis for ulcerative colitis do not have a consistent change in permeability(85).

Other poorly understood immunologic factors contributing to Crohn's may be affected by intestinal surgery. Beta-defensins are peptides that contribute to nonspecific mucosal defense against bacteria and have been proposed as mediators of inflammation in inflammatory bowel disease(86). There is as yet no research on their role following intestinal surgery but they are known to be increased in tears following ocular surgery(87). While this is far from a clear association, further studies may provide interesting insights.

Whatever the answers to the many remaining questions, further research and understanding of the inflammatory process that takes place following surgery in Crohn's patients is crucial to the development of more effective therapies.

ANATOMICAL CONSIDERATIONS AND THE EFFECT OF FECAL FLOW

Intestinal surgery can result in changes to Crohn's expression through anatomical as well as immunological alterations. Segments of bowel excluded from flow of the fecal stream will generally remain free of disease, a phenomenon that can be seen when the fecal flow is purposefully diverted from colon and ileum(88) or when an anastomotic technique is used that excludes a pouch of ileum(89). The essential nature of the fecal stream for disease expression was confirmed when it was

demonstrated that disease could be induced by infusing intestinal fluid into an excluded ileal loop(90). Thus surgery can interact with Crohn's disease by altering the balance of inflammatory and regulatory cytokines, but also by grossly altering the balance of enteric bacterial flora.

The exclusion of a segment of intestine to treat Crohn's of that segment has been used to therapeutic advantage in certain circumstances. Patients with severe colitis or perianal fistulizing disease that are refractory to medical therapy can have improvement following a temporary diverting ileostomy. The obvious limitation of this technique is that disease will generally recur following reanastomosis, and the only advantage over a resection of the involved segment is the possibility of reestablishing bowel continuity.

The attempt to maintain bowel continuity and continence as opposed to ileostomy has led to the introduction of new surgical techniques. Ileal pouch-anal anastomosis (IPAA) is a technique that has gained widespread acceptance following total colectomy for ulcerative colitis and familial adenomatous polyposis. The procedure involves the formation of an ileal pouch in order to increase capacitance of the terminal ileum, then anastomosis to the anus, where physiologic continence mechanisms can be retained. Because total colectomy for ulcerative colitis is a curative procedure, but Crohn's disease can occur in the pouch, initial experience with the procedure in Crohn's disease was often obtained inadvertently when a presumed case of ulcerative colitis developed small bowel manifestations confirming

underlying Crohn's. Higher pouch failure rates in Crohn's patients, often as a result of recurrent inflammation, led to the widespread conclusion that IPAA was not a viable option for Crohn's, but later results suggested that patients whose pouches remain intact have functional outcomes comparable to ulcerative colitis patients(91-93). Nevertheless, the intentional use of IPAA in patients with Crohn's currently remains confined to only certain centers.

IPAA surgery has been interesting in the study of inflammatory bowel disease pathogenesis for other reasons as well. When the surgery is done for ulcerative colitis, pouch inflammation (pouchitis) is a troublesome problem that occurs in 50% of patients after 10 years. Although it is idiopathic, research reveals reduced counts of lactobacilli and bifidobacteria within the pouch (94). The clearly altered bacterial profile in pouchitis, and the fact that it can be treated effectively by both probiotics (95) and antibiotics(96) makes it the clinical setting that most clearly demonstrates evidence of an altered fecal flora as a pathogenic mechanism in inflammatory bowel disease.

MEDICAL PROPHYLAXIS AGAINST POSTOPERATIVE RECURENCE

Since many patients undergoing surgery for Crohn's disease will have had a serious or dramatic presentation of their disease, there is no more important setting for the

study of medical prophylaxis against recurrent Crohn's. A summary of evidence for different treatment interventions is reported in Table 1.

BLOOD TRANSFUSION

The fact that blood transfusion has been known to have an important immunosuppressive effect in some settings led to studies suggesting that perioperative blood transfusion might decrease the later possibility of relapse following resection(97). Unfortunately, the pooling of data from multiple trials did not support any beneficial effect(98).

5-AMINOSALICYLATE

Sulfasalazine was the agent used in most early trials of 5-aminosalicylate (5-ASA) for maintenance of surgically-induced remission in Crohn's disease, but trials have since included other preparations in which the active agent is 5-ASA. Details of the randomized controlled trials studying 5-aminosalicylates are presented in Table 2. Data from the National Cooperative Crohn's Disease Study which included 48 postoperative patients, 15 of which were randomized to sulfasalazine, did not demonstrate improved times to recurrence in those receiving the 5-ASA agent(99). Another study of 66 patients randomized to sulfasalazine (3g/day) or placebo following resection did not demonstrate a difference in clinical recurrence rates between the two groups at one year of follow up (13% in the treatment group and

15% in the placebo group), but a difference became apparent when 26 patients were followed for another six months (13% recurrence in the treatment group versus 45% in the placebo group)(100). These more promising preliminary results were followed by a larger study of 232 patients followed for three years, in which postoperative patients were randomized to receive sulfasalazine or placebo(101). Recurrence rates at 1 year were 16% in the treatment group and 28% in the placebo group, a difference that was statistically significant, but at three years the recurrence rates were similar. In summary, results demonstrating a positive effect for sulfasalazine are not consistent between trials, but recurrence rates at one year may be improved, an effect that is statistically significant when data is pooled between studies(102).

Several studies have now examined other 5-ASA containing preparations for medical prophylaxis against postoperative recurrence. The largest was conducted as part of the European Cooperative Crohn's Disease Study(103). 318 postoperative patients were randomized to receive placebo or mesalamine (Pentasa 4g/day) and were followed for 18 months. At the conclusion of the study, clinical recurrence occurred in 24.5% of treated patients, and 31.4% in the placebo group. This trend was not statistically significant ($p=0.10$), but post hoc analysis revealed a significant effect in the subgroup of patients with isolated small bowel disease.

The second-largest randomized controlled trial of mesalamine in this setting was conducted by McLeod et al(104), and randomized 163 patients to mesalamine (3g/day) or placebo. They found a risk ratio for recurrence of 0.628 favoring the

treated group and, strangely, found the greatest treatment effect in patients with isolated colonic disease.

More recently, one randomized controlled trial evaluated mesalamine at a dose of 4g/day as compared with a dose of 2.4g/day, and found no difference in rates of clinical recurrence at 12 months(105). Another recent randomized controlled trial, designed primarily for the evaluation of 6-mercaptopurine, evaluated mesalamine as compared with 6-mercaptopurine or placebo, but did not show any significant difference between groups in terms of clinical recurrence at two years postoperatively(106).

Other double-blinded, randomized controlled trials of mesalamine in this setting have yielded inconsistent results(107-109), but a majority of studies support the efficacy of 5-ASA for maintenance of surgically-induced remission, and a meta-analysis of all randomized trials fully published by 1997 suggested that mesalamine decreases the risk of postoperative recurrence by approximately 13%(110).

In summary, the weight of evidence supports some efficacy of 5-ASA in the maintenance of postsurgical remission in Crohn's disease. Unfortunately, different preparations, doses, and durations of follow up are used between studies, and in some studies showing no effect, therapy was not provided for a prolonged interval following surgery(103). Overall, the effect of mesalamine is likely mild.

STEROIDS

In remissions achieved without surgery, traditional corticosteroids have no benefit as maintenance therapy(111). A study of sulfasalazine combined with prednisolone for the maintenance of postoperative remission has also failed to demonstrate a benefit in that setting(112). Budesonide, a corticosteroid with targeted delivery to the terminal ileum and proximal colon, has been studied in a randomized controlled trial in which no benefit of therapy was detected(113). In summary, corticosteroids are not appropriate for maintenance therapy of Crohn's disease.

ANTIBIOTICS

Antibiotics have been widely used in the treatment of active Crohn's disease, but only two randomized controlled trials are available to support their use for maintenance of postoperative remission(114, 115). The first study by Rutgeerts *et al* assigned postoperative patients to 20mg/kg/day of metronidazole for a total of 12 weeks(114). One year recurrence rates were 25% for the placebo group, and only 4% for the treated group, a difference that was statistically significant. After three years, however, the endoscopic recurrence rates were the same between the two groups, and clinical recurrence rates showed only a nonsignificant trend favoring therapy.

The second study published by Rutgeerts and colleagues further explored the use of nitroimidazole antibiotics in this setting(115). Eighty postoperative patients were randomized to placebo or ornidazole (Tiberal; Roche, Basel, Switzerland) 500mg

twice daily for one year. The primary endpoint was clinical recurrence one year after surgery, and a significantly lower rate was found in the treated group than in those receiving placebo (37.5% in the placebo group versus 7.9% of patients in the ornidazole group). Ornidazole was used because it was thought to have side effects qualitatively similar to those of metronidazole, but of decreased intensity. Unfortunately, in this study over 30% of patients in the ornidazole group dropped out as a result of side effects, the most common being nausea, vomiting and metallic taste. All of these have been troublesome in the long-term use of metronidazole as well.

These two well-designed studies support the effectiveness of nitroimidazole antibiotics in the maintenance of surgically-induced remission of Crohn's disease, but leave us without clear guidance in terms of optimal therapy, because of high incidence of side effects, and a lack of data to indicate optimal dosing or duration of therapy.

IMMUNOMODULATORS

6-mercaptopurine (6-MP) and azathioprine (AZA) are effective in the maintenance of medically induced remission of Crohn's disease(116). Data supporting their use in the postoperative setting is limited, but retrospective analyses have suggested a possible benefit(117, 118). Two recently published randomized controlled trials added to the available data. Hanauer *et al* assessed 131 postoperative patients

randomized to either placebo, mesalamine 3g/day or 6-MP 50mg/day and followed for two years(106). Rates of endoscopic and clinical recurrence at multiple time points were assessed, and generally showed nonsignificant trends favoring 6-MP. For example, clinical recurrence rates at 2 years were 77% for placebo, 58% for mesalamine, and 50% for 6-MP, with the 95% confidence intervals overlapping for all groups, but survival analysis showing a difference between 6-MP and placebo ($p=0.045$). The authors felt the study illustrated the efficacy of 6-MP as compared with placebo, but their conclusions have been debated in subsequent editorials(119, 120). It should also be noted that the dose of 6-MP they used was relatively low as compared with doses known to be effective in other clinical settings.

The second study, by Ardizzone *et al*, compared AZA at a dose of 2mg/kg/day to mesalamine or placebo in an unblinded, prospective randomized study of 142 patients lasting two years. Two-year clinical recurrence rates were 28% for mesalamine and 17% for AZA, a difference that did not reach statistical significance(121).

In summary, data does not strongly support the maintenance efficacy of AZA or 6-MP in the postoperative setting, but does show trends in their favor. Because of the stronger evidence supporting the use of AZA or 6-MP in the setting of medically-induced remission, even authors highly critical of the existing data acknowledge that the use of thiopurines is likely warranted in high risk postoperative patients(119).

PROBIOTICS

The role of intestinal bacteria in the pathophysiology of Crohn's disease has led to the use of beneficial enteric bacteria as therapeutic agents. Some experience is available in the setting of postoperative maintenance therapy. One randomized controlled trial of *Lactobacillus* GG *casei* subspecies *rhamnosus* (1.2×10^{10} cfu daily) or placebo failed to show any benefit in terms of clinical recurrence rate at one year(122). A second study, by Campieri *et al* (123) reported, in abstract form, that a combination of antibiotic and the probiotic mixture (VSL#3®) treatment was efficacious in prevention of the postoperative recurrence of Crohn's disease when compared to mesalamine. Forty patients were randomized to receive either rifaximin (1.8 g per day) for 3 months followed by VSL#3® (6×10^{11} cfu per day) for 9 months, or mesalamine (4 g per day) for 12 months. After 1 year, the antibiotic/VSL#3® group had an endoscopic recurrence rate of 20% compared to 40% in the mesalamine group ($p < 0.05$). These endoscopic recurrence rates at 1 year with an antibiotic followed by VSL#3® are similar to those that have been previously described with metronidazole alone(114), raising the question of whether the benefit was provided by the antibiotics.

In summary, probiotics are promising agents, but have not demonstrated efficacy in the setting of postoperative Crohn's disease maintenance.

THE NECESSITY FOR FURTHER RESEARCH

Surgery is an important therapeutic tool for Crohn's disease, but its optimal use is limited by our understanding of the disease process. Studies of the natural history of postoperative Crohn's disease have already had positive impact on patient care, for example, by leading to the replacement of traditional wide resections by bowel-sparing procedures. Unfortunately, there remain large gaps in our knowledge that preclude the identification of patients at high risk for postoperative relapse and the optimal treatment for maintenance of postoperative recurrence.

Ongoing research has identified several intestinal tissue markers that may allow earlier diagnosis of Crohn's disease relapse, including decreased diamine oxidase(124), increased interleukin 5 expression(125), increased interleukin 6 expression(126) and increased phospholipase A2 activity(127). Even more exciting is the possibility that perioperative measurements could be used to predict postoperative recurrence. Meresse and colleagues demonstrated that ileal IL-10 mRNA concentration at the time of surgery correlates inversely with the subsequent risk of relapse, but found no relationship with perioperative levels of tumor necrosis factor alpha or Il-1 beta(128).

Of course, to take advantage of an enhanced ability to predict and detect postoperative recurrence, we must have effective therapies to offer patients. While evidence remains controversial for the use of thiopurines as postoperative maintenance, it could be that patients at highest risk of relapse would be most likely to benefit. The development of new agents for Crohn's disease maintenance is also

promising. Infliximab has yet to be studied in the postoperative setting, but it may be another therapeutic option in the future.

Studies of postoperative Crohn's disease in humans remain fundamentally limited by the complexity of the patients' illnesses and by the necessity to provide optimal patient care. Because our knowledge remains flawed at such fundamental levels, the use of an animal model could potentially answer many crucial questions. Ideally, it could be used to measure the immune changes that occur at surgical sites, and to identify defects in the responses of IBD animal models. Once identified, correction of these defects could be the key to targeting postoperative IBD therapy to correct the crucial abnormalities. The experiments here proposed require a surgical model that isolates the effect of intestinal surgery on immune physiology, and a suitable animal model of IBD.

ANIMAL MODELS OF INTESTINAL TRANSECTION AND ANASTOMOSIS

A surgical technique for the transection and reanastomosis of mouse and rat small bowel has been developed for the study of small bowel resection. The technique is facilitated by the use of a rapidly digested internal splint and indigestible but absorbable sutures. It has been further refined in terms of suture sizes and abdominal wall closure technique. Experiments designed to examine the effect of the surgical insult itself could use a transection and reanastomosis without any intervening resection, since this would isolate for the effect of the incisional injury. 15-20%

subject mortality has been previously described for animals undergoing massive small bowel resection. This could be expected to be an upper estimate for animals undergoing transection and reanastomosis with no bowel resection(129, 130).

THE IL-10 DEFICIENT MOUSE MODEL OF INFLAMMATORY BOWEL DISEASE

Many animal models are available for the study of Crohn's disease, but none are perfect. The cotton-top tamarin can develop relapsing/remitting colitis mimicking human IBD, but like human IBD its precise genetic etiology has not been determined, making it a complicated subject for study.

The IL-10 deficient mouse is a disease model that has undergone considerable study, and several features make it an attractive subject for experimentation related to human IBD. Like human Crohn's disease, the phenotype developed by IL-10 deficient mice includes transmural intestinal inflammation, and inflammation that can span the entire digestive tract(131, 132). Despite the single-gene etiology of enterocolitis in IL-10 knockout mice, the model acquires downstream features of Crohn's disease that make it more than a simple study on the absence of IL-10. NF-Kβ, known to be important in the pathophysiology of human Crohn's disease, is increased in the lamina propria of IL-10 deficient mice(133), and other mediators of human IBD, such as TNFα and IFNγ are also involved in the colitis of IL-10 knockout mice(132, 134). Enterocolitis developed by IL-10 knockout mice also takes

on a dependence on environmental factors similar to human Crohn's disease, in that it is highly dependent on the quantity and quality of their intestinal flora(135, 136). That the gene abnormality in IL-10 knockout mice triggers a cascade of factors giving it a complex phenotype similar to human IBD is supported by the fact that IL-10 therapy can prevent, but not abort ongoing colitis in affected animals(131), and may be due to the fact that IL-10 is involved in the generation of regulatory T-cell lines that suppress colitis(133, 137).

When combined with the fact that laboratory mice have been previously useful for experimentation in surgical models, the favorable physiologic features of the IL-10 knockout mouse make it an ideal subject for the study of postoperative Crohn's disease.

CONCLUSION:

Improved therapy of postoperative Crohn's disease requires enhanced understanding of its pathophysiology in order to more effectively target immunological defects inherent in the disease. Intestinal transection and reanastomosis experiments in the IL-10 deficient mouse offer a unique opportunity to explore these issues.

CHAPTER 2

EXPERIMENTAL DESIGN

HYPOTHESIS

Intestinal surgery predisposes to inflammatory bowel disease recurrence. There are several mechanisms by which this could occur:

1. The inflammatory reaction associated with normal wound healing could be deregulated and of increased magnitude in individuals predisposed to inflammatory bowel disease.
2. Intestinal surgery could result in increased intestinal permeability, thus accentuating one factor thought to be crucial in the pathogenesis of Crohn's disease expression.
3. The normal state of immune tolerance to enteric bacteria may be broken by immune activation at the time of surgery.

Each of these mechanisms will be explored by testing the following hypotheses:

1. The postoperative state will be associated with a local accumulation of proinflammatory cytokines.

2. Postoperative mice will have higher intestinal permeability than animals that underwent a sham procedure.
3. Postoperative mice will have higher systemic immune reactivity to stimulating factors than those who underwent a sham procedure.

EXPERIMENTS

In order to test the hypothesis, the following sequence of procedures and experiments were carried out:

1. Previously described techniques for intestinal transection and anastomosis were adapted to Sv/Ev129 mice. A sham surgery was devised in order to isolate for the effects of surgical incision on the operated animals. Postoperative mortality was measured, and growth curves taken. This was compared to results in IL-10 knockout mice.
2. Following surgery, intestinal histology was measured, and indicators of injury and inflammation were graded using a standardized scoring system. Extent of injury in millimeters was measured at several postoperative time points.
3. Intestinal permeability was assessed postoperatively in operated and sham animals using an indirect method (bacterial translocation), and a direct *in vitro* method (Mannitol flux).
4. The inflammatory response was quantified and anatomically mapped by measuring levels of cytokines and/or their RNA at varying distances from the anastomotic site and at several pre and postoperative timepoints.

5. Systemic immune responsiveness was assessed by isolating splenic lymphocytes from postoperative surgical and sham animals. Splenocytes were stimulated with broad stimulants and a variety of bacterial sonicates, and their response was measured in terms of inflammatory cytokine production and cell division.
6. To follow up results of stimulation experiments on splenocytes, additional splenocytes were isolated postoperatively and analyzed by flow cytometry to determine the varieties of spleen cells present.

CHAPTER 3

MATERIALS AND METHODS

MICE

All experiments were approved by the animal ethics committee at the University of Alberta hospital. Wild-type mice (WT) were Sv/Ev129, and Interleukin-10 knockout (IL-10KO) mice were transgenic mice on the same background.

As preliminary experimentation indicated that IL-10KO mice showing any signs of active inflammatory bowel disease would not survive the operation, operations were performed on mice that were in the preclinical age group of 10-14 weeks.

To maximize the tissue available from operated mice, experiments were performed on four groups of animals:

Group #1: In the first group of mice the livers and spleens were used for bacterial translocation experiments; half of the small bowel (divided longitudinally) was used for histology, and half for cytokine RNA measurement by polymerase chain reaction (PCR). Cecums of these mice were also kept for quantitation of cytokines by ELISA. In this group, six WT surgical and six WT sham mice were used at each of the timepoints: 1, 2, 4, 8 and 16 days postoperative. Six WT mice in total were sacrificed as controls at day 0, and they had no intervention. These experiments were replicated

in age-matched IL-10KO mice with three surgical and three sham mice at timepoints: 2, 8 and 16 days postoperative. Three IL-10KO mice were sacrificed as day 0 controls. Cytokine mapping by PCR had proven cumbersome and yielded equivocal results in WT mice, so these experiments were not replicated in IL-10KO. Mice who died perioperatively were replaced with new mice.

Group#2: These mice were only used for small intestinal permeability experiments in Ussing chambers. Six WT surgical and six WT sham mice were used for these experiments, and were assessed at day 2. These experiments were replicated with the same number of IL-10KO mice.

Group #3: Mice whose small bowels and spleens were used for lymphocyte stimulation experiments were not used for other experiments. Four WT surgical and four WT sham mice were used for these experiments, which were done on postop day 7. Again, these experiments were replicated in the same number of IL-10KO mice. As described in the next paragraph, some of these experiments were replicated with Group #4 mice. For this reason, experiments involving splenocyte stimulation by ConA, anti-CD3 and *E. cloacae* are each based on groups of 7 surgical and 7 sham mice.

Group #4: These mice had their small bowels used for permeability experiments in Ussing chambers at day 7. Their spleens were harvested for flow cytometry. Six WT mice were used (three sham and three surgery), and experiments were replicated in

the same number of IL-10KO. At the time that this group of mice underwent surgical procedures, dramatic results had been obtained for group #3 mice in some splenocyte stimulation experiments. The spleens of some group #4 mice were therefore used to replicate splenocyte stimulation experiments.

Following surgery mice were weighed daily and monitored for signs of discomfort. All mice used for experiments were vigorous and thriving at the time of sacrifice. Mice were sacrificed by cervical dislocation.

SURGICAL PROCEDURE

The operative procedure of intestinal transection/reanastomosis was adapted from the sham procedure in previous studies of small bowel resection in rodents(129, 130).

No preoperative bowel preparation was done, and no perioperative antibiotics were used.

Animals undergoing surgery were anaesthetized with inhaled isofluorane. The abdomen was prepped with providone/iodine solution and the entire procedure was conducted using clean technique. The abdominal cavity was accessed through a midline incision. The ileocecal region was then identified, and a single incision was made through the entire small bowel at a site one centimeter proximal to the ileocecal junction. Care was taken not to damage the mesenteric vessels, but no other

hemostatic techniques were used, and mesenteric vessels were not ligated. A digestible carbohydrate stent was inserted into the small bowel [*Primo* spaghettini, Kraft Canada], and the bowel was anastomosed using nonabsorbable 10-0 nylon monofilament suture [*Sharpoint*, Surgical specialties corporation, Reading, PA]. A total of six to eight interrupted sutures were used. The peritoneum was then irrigated with warm, sterile normal saline and the peritoneum and abdominal wall were closed with 6-0 braided silk sutures [ETHICON, Johnson and Johnson medical products, Markham, Ontario]. One to three interrupted sutures were used to close the peritoneum, and a continuous suture was used for the skin. Animals received a single subcutaneous dose of buprenorphine (0.1mg/kg) as postoperative analgesia at the conclusion of the operation.

Animals undergoing sham operation had identical anesthesia and abdominal incisions. Their bowel was mobilized, their abdomen irrigated, and the abdominal wall closed as in the surgical animals, the difference being that they underwent no bowel incision or anastomosis.

Both surgical and sham animals were allowed to drink water postoperatively, but food was withheld until 24 hours later. Mice were weighed daily and monitored for signs of discomfort (eg. piloerection, rectal prolapse, decreased spontaneous movement). As early experience demonstrated that mice not thriving on the first postoperative day were unlikely to fully recover, all mice with signs of pain at any time on or after postoperative day 1 were sacrificed. These sacrificed mice were autopsied, but no

further data was used from their tissues. All mice used for experiments were vigorous and healthy-looking until the time of sacrifice.

HISTOLOGY

Small bowels used for histology and cytokines were divided longitudinally, with half of the tissue taken for each set of experiments. Histology samples were cut and stained with standard hematoxylin and eosin, and mounted in a configuration demonstrating which end was proximal, and which distal. A single pathologist interpreted all results, and was blinded to the time point and surgical group of the tissue under study. The bowel was examined for features of inflammatory bowel disease, and postoperative injury was graded using the Ehrlich-Hunt numerical score, which measures injury according to six cellular and tissue parameters(138).

BACTERIAL TRANSLOCATION

For the purpose of measuring bacterial translocation across the intestinal wall, mice had their spleen and the left lobe of their liver removed, and all steps were performed in sterile fashion. Each organ was completely homogenized using two frosted glass microscope slides, then suspended in 1mL of sterile PBS buffer. 100μL of solution was then plated onto Brain Heart Infusion agar and incubated at 37°C. The number of bacterial colonies present on the plate was counted 24 hours later. Samples that became grossly contaminated during the procedure were discarded.

INTESTINAL PERMEABILITY

Following sacrifice of experimental mice, their small bowels were removed. The average small bowel in our animals had a length of approximately 21cm. For postoperative day 2 experiments, we took three segments, each 5cm in length, proximal to the site of surgical anastomosis. As the majority of the small bowel was used, we labeled the three sites somewhat arbitrarily as "ileum", "distal jejunum" and "proximal jejunum" in order from distal to proximal.

Experiments conducted on postoperative day 7 used less tissue samples, as these mice were also being used for splenic experiments that required large numbers of mice to be sacrificed in a short period of time. These mice each had two samples of small bowel used for experiments, both taken from as closely adjacent to the ileal anastomotic region as possible.

The segments of bowel were measured for Mannitol flux in Ussing chambers, as has been described previously(139). In brief, the tissue was mounted in Lucite chambers exposing serosal and mucosal surfaces to Krebs buffer maintained at 37°C. Fructose (10mMol/L) was then added to both serosal and mucosal sides. 1mmol/L of mannitol with 10 μCi [H^3] was added to the mucosal side. The spontaneous transepithelial potential difference (PD) was determined, and the tissue was clamped at zero voltage by continuously introducing an appropriate short cicuit current (Isc) with an

automatic voltage clamp (DVC 1000 World Precision Instruments, New Haven, CT), except for 5-10 seconds every 5 minutes when PD was measured by removing the voltage clamp. Tissue ion conductance was calculated from PD and Isc according to Ohm's law.

CECAL CYTOKINE MESUREMENT

Colon organ cultures were prepared from 6 day-0 mice, and 6 surgical and 6 sham-operated mice at each of the time points: 1, 2, 4, 8 and 16 days postoperatively for WT mice. For IL-10KO mice, 3 day-0 mice were used, as well as 3 surgical and 3 sham-operated mice from each of the time points: 2, 8 and 16 days. Colons were removed, flushed with cold PBS, and cut into 2mm squares. Each square was washed and suspended in tissue culture wells (Falcon 3046; Becton Dickinson Labware, Lincoln Park, NJ) in RPMI-1640 supplemented with 10% fetal calf serum, 50mM 2-β-mercaptoethanol, penicillin (100 U/mL), streptomycin (100 U/mL), and the presence or absence of lipopolysaccharide (LPS). Cultures were incubated at 37°C in 5% CO_2 for 6 hours for measurement of TNF-α and 24 hours for measurement of IFN-γ . Supernatants were harvested and stored at -70°C for analysis of cytokine levels. TNF-α and IFN-γ levels in cell supernatants were measured using enzyme-linked immunosorbent assay (ELISA) kits (Medicorp, Montreal, Quebec).

CYTOKINE-SPECIFIC ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

For the assessment of cytokine production by spleen cells, a quantitative cytokine specific sandwich ELISA was performed on 96-well, high-protein-binding ELISA plates (Costar, Corning Inc., Corning, NY, U.S.A.). The following antibodies were used: anti–IFN-γ clone R4–6A2 and anti–IL-10 clone JES5–2A5 (as capture antibodies) and biotinylated anti–IFN-γ clone XMG1.2 and anti–IL-10 clone JES5–16E3 (as detection antibodies). All antibodies and recombinant cytokine standards were purchased from PharMingen (Mississauga, Ontario, Canada) and used at pretitered concentrations to give optimal results. The color was developed using streptavidin peroxidase (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, U.S.A.) and 3,3',5,5'-tetramethylbenzidine (TMB) (SIGMA, St. Louis, MO, U.S.A.), and the reaction was stopped with 1 M H_2SO_4. The plates were read in an ELISA reader (SLT Instruments, Salzburg, Austria), and the amounts of cytokine in individual samples were assessed against the standard curve.

REVERSE-TRANSCRIPTION POLYMERASE CHAIN REACTION FOR CYTOKINES

Total RNA from small bowel segments was isolated using TRIzol (Gibco, Burlington, Ontario, Canada) following the manufacturers instructions. mRNA was reverse transcribed and amplified using the polymerase chain reaction (PCR) using β-actin as an endogenous control as previously described(140). The primer sequences used for PCR are shown in Table 3. They were based on murine mRNA sequences for the

respective cytokines obtained from GenBank and used to design intron-spanning primers. Polymerase chain reaction products were separated on 2% agarose gels containing ethidium bromide and visualized and photographed under UV light. Quantification by densitometry was performed using a Digidoc gel doc system (UVP, Upland, CA). In order to quantitatively map the proportional change in cytokine quantities at variable distances from the anastomotic site, the five small bowel samples from individual animals underwent the same PCR reactions, and were run on the same gels. The 1cm segment on the caudal side of the anastomosis (between the surgical site and the cecum) was used as an internal control, and quantities of RNA in the remaining (orad side) small bowel were expressed as a proportion of the cecal-side segment.

IN VITRO SPLEEN CELL REPONSIVENESS TO BACTERIAL ANTIGENS

Spleens were removed and minced between the frosted ends of slides to prepare a single-cell suspension in complete RPMI with 10% fetal calf serum (FCS). Red blood cells were lysed by osmotic shock and lymphocytes were placed into the wells of 96-well plates at a concentration of 2×10^5 per well. Bacterial sonicates, at a concentration of 50μg protein/mL, and the T-cell mitogen ConA (SIGMA, St. Louis, MO, U.S.A.), at a concentration of 1 μg/mL, were added. Controls included plate bound anti-CD3ε clone 145–2C11 (PharMingen Canada, Mississauga, Ontario, Canada) and medium alone. After 48 hours incubation at 37°C in a humidified

incubator at 5% CO_2, the plates were centrifuged and the amounts of interferon gamma (IFN-γ) and IL-10 in the supernatants were quantified by enzyme-linked immunosorbent assay (ELISA).

FLOW CYTOMETRIC ANALYSIS

Flow cytometric analysis of spleen cells from mice that had undergone ileal surgery and sham-operated mice were performed according to standard procedures. Briefly, red blood cell-depleted splenocytes were suspended in PBS staining buffer containing 2% bovine serum albumin (BSA) and 0.02% NaN_3 at a concentration of 1×10^6 cells/ml. Directly conjugated pre-titered monoclonal antibodies were added to cell suspension and incubated for 20 minutes at 4°C. The following monoclonal Antibodies were used: anti-CD3ε (clone 145-2C11) coupled to APC, B220 (clone RA3-6B2) coupled to FITC, and anti-CD11b (clone M1/70) coupled to PE. All monoclonal antibodies were purchased from BD Biosciences (Mississauga, ON). Following staining, the samples were washed three times in PBS and resuspended in Fix buffer, consisting of PBS with 1% paraformaldhyde and 0.02% NaN_3. Samples were stored at 4°C before cytometric analysis. The analysis was performed on a BD FACScalibur™ flow cytometry system in the University of Alberta Heritage Research Building.

STATISTICS

Error bars on all figures are expressed as standard error. For permeability experiments and cecal cytokine measurements, two-sided p-values were calculated using the Mann-Whitney U test. P-values for histological data are calculated from two-tailed t tests.

All experiments were conducted primarily as comparisons between surgical animals and their sham-operated counterparts. All p-values refer to this comparison.

CHAPTER 4

RESULTS

SURGICAL OUTCOMES

Postoperative mortality results are summarized in Table 4.

A total of 97 WT mice were used. 48 mice underwent surgery, of which 5 were sacrificed postoperatively because of failure to thrive, for a mortality rate of 10%. Autopsies revealed operative site complications of leakage and/or obstruction in those mice doing poorly postoperatively. 43 mice underwent sham procedures with no mortality, and 6 mice were day 0 controls.

A total of 66 IL-10KO mice were used. Subjectively, surgery was no more challenging than in WT mice, and tissues had similar characteristics to their WT counterparts. 36 IL-10KO mice had intestinal surgery, and 17 died postoperatively for a total mortality of 47%. Autopsies revealed operative site complications in only 10-15% (mild leakage being occasionally equivocal), with the remainder of ill animals having no evident abnormalities. The excess operative mortality in IL-10KO mice was therefore speculated to be a result of sepsis, or systemic inflammatory response syndrome independent of infection. At the time of sacrifice for data, the IL-10KO mice did have subjectively poorer healing than their WT counterparts, with weaker anastomotic strength. 27 IL-10KO animals underwent the sham procedure, with 4 deaths, for a total of 15% mortality. 3 animals were day 0 controls.

Following surgery, mice typically lost weight for two to four days before gradually regaining. Sham-operated mice typically had little weight change. In general, IL-10KO mice recovered more slowly, and occasionally continued losing weight, even while looking vigorous. These trends are indicated on Figures 1-4, which represent the growth curves of all mice used for experiments on postop day 16.

HISTOLOGY

Histological changes in WT mice extended an average distance of 1.69mm from the anastomotic site [95% confidence interval: (1.30, 2.08)], as compared with IL-10 knockout mice, in which they extended an average of 5.75mm [95% confidence interval: (4.38, 7.12)] ($p<0.001$).

As measured by the Ehrlich-Hunt score, the predominant pattern of histological changes over time was an early neutrophilic response that decreased with time postoperatively, and a later recruitment of fibroblasts. This is illustrated for WT mice in Figure 5. Changes in structural components are shown for WT mice in Figure 6, and demonstrate an early increase in collagen content. Cellular changes for IL-10KO mice are shown in Figure 7. As can be seen, WT mice seemed to mount a more intense fibroblastic response than IL-10KO mice. Structural components for IL-10KO mice are illustrated in Figure 8, and demonstrate a later increase in collagen

content than that seen in WT mice. For clarity, not all Ehrlich-Hunt parameters have been discussed and illustrated.

Representative photographs of operative sites are shown in Figures 9-14.

BACTERIAL TRANSLOCATION

In WT mice (Figures 15 and 16), both liver and spleen had little to no evidence of culture at baseline or after postoperative day 4. Among the sham operated animals, the pattern was one of increased cultures generally only on day 1. Surgical animals in general had positive cultures on days 1 to 4, with similar patterns in liver and spleen cultures.

In IL-10KO mice, no positive cultures were seen on liver or spleen in any sham-operated animals. In surgical animals, no positive cultures were seen on days 0 or 16. A large number of cultures were present in spleens of the day 2 surgical group. Wide variation was present between animals, but the overall pattern was similar to wild type mice, as illustrated for liver cultures in Figure 17 and spleen cultures in Figure 18.

INTESTINAL PERMEABILITY

Intestinal permeabilities in 2-day postoperative mice, as measured by mannitol flux, are summarized in Figure 19. At all individual anatomical sites, permeabilities were lower in the surgical animals than those who underwent sham procedures. This pattern was seen in both WT and IL-10KO mice. Permeabilities at different anatomical locations in the same animal groups were similar.

In WT mice, comparisons between surgical and sham animals at individual anatomical points were not statistically significant, but the comparison between average permeabilities was significant ($p<0.05$). In IL-10KO mice, comparisons were significant ($p<0.05$) between sham and surgical animals at the proximal jejunal site and for average permeability, but were not significant at other individual sites.

Permeability data on postoperative day 7 is summarized in Figure 20. At this time point, no data was taken for individual anatomic locations; all data was collected from the ileum. In both WT and IL-10KO mice, permeabilities were now similar between surgical and sham-operated mice. No comparisons were statistically significant.

CECAL CYTOKINES

Among wild-type mice, levels of cecal IFN-γ and TNF-α had high variation between individual animals, but there was no significant differences between surgical and sham-operated animals at any time point (see Figures 21 and 22). Levels of cecal

cytokines in IL-10KO mice varied so highly between individual mice that no clear trends could be observed. Data for IL-10KO mice are not shown.

SMALL INTESTINAL CYTOKINES

Results of cytokine mRNA measurements for WT sham-operated mice are shown in Figures 23-25, and demonstrate no pattern of higher cytokine expression for any of the measured cytokines at any anatomic location.

In WT surgical mice (Figures 26-28), TGF-β (Figure 26) and TNF-α (Figure 27) can clearly be seen to peak around the operative site at all time points. In contrast, IFN-γ has not peaked near the operative site on days 2 or 8, but does appear to be elevated near the anastomotic site by day 16 (Figure 28).

SYSTEMIC IMMUNE RESPONSIVENESS

Attempts to isolate small bowel lymphocytes did not succeed in yielding enough cells to conduct these experiments, so all data below is reported for splenocytes.

Splenic lymphocytes isolated from WT and IL-10KO animals that underwent surgery or sham procedures were stimulated with anti-CD3 or ConA, and their responsiveness was measured as production of IFN-γ. The results are summarized in Figure 29.

Stimulation by anti-CD3 resulted in similar levels of IFN-γ production between surgical and sham-operated animals in both WT and IL-10KO animals. IL-10KO mice produced a mildly greater quantity of IFN-γ than WT in both surgical and sham-operated groups.

Stimulation by ConA resulted in dramatically less production of IFN-γ by postsurgical mice than sham-operated animals in WT, a difference that was statistically significant. Among IL-10KO mice, this pattern was not present; IL-10KO and WT mice produced similar levels of IFN-γ.

Results of experiments illustrating splenic lymphocyte responsiveness to bacterial sonicates are illustrated in Figure 30. No qualitatively appreciable differences were seen between surgical and sham-operated mice with any sonicate, except perhaps a lesser responsiveness towards *E. cloacae* in operated mice, a nonsignificant trend that mirrored the changes seen with ConA stimulation.

Splenocyte stimulation experiments were replicated in WT and IL-10KO mice using cellular proliferation, as measured by radiolabelled thymidine incorporation, as an indicator of response to stimuli (Figures 31 and 32). Once again, no appreciable difference was present between sham and surgical animals when stimulated by a variety of bacterial sonicates. In WT animals, stimulation by both ConA and anti-CD3 resulted in less thymidine incorporation in animals that underwent surgery than

in those who had undergone a sham operation. In IL-10KO mice, results were similar in surgical as compared with sham-operated mice.

In order to help explain the lesser responsiveness to ConA stimulation seen in postoperative WT mice, as compared with their sham-operated counterparts, we measured quantities of IL-10 produced following stimulation with ConA, anti-CD3 and E. cloacae. Results are shown in Figure 33, and demonstrate that postoperative WT mice have a nonsignificant trend towards higher Il-10 production than sham-operated mice, when exposed to all three stimulants. As expected, no IL-10 production was observed by the splenocytes of IL-10KO mice.

FLOW CYTOMETRY

Flow cytometry was carried out to determine whether changes in splenocyte responsiveness were a result of altered cell populations or of a true alteration in T-cell responsiveness. Results from flow cytometry of postoperative day 7 splenocytes from surgical and sham-operated mice in both WT and IL-10KO are illustrated in Figure 34. No statistically-significant differences were found between proportions of cells present between sham and surgical animals in WT or IL-10KO, but there was a trend among WT mice towards decreased T-cells (CD3+) and increased macrophages (CD11b+) in operated animals.

CHAPTER 5

DISCUSSION

EXPERIMENTAL MODEL

The study of inflammatory bowel disease is complicated by the fact that so many biological systems interact in order to produce disease. Nowhere is this fact more obvious than in the study of postoperative Crohn's disease where the body's immune system is involved in a war on several fronts, against relatively pathogenic intestinal bacteria, against a surgical defect requiring closure, and perhaps against its own failings that have led to IBD in the first place. The natural history of Crohn's disease makes it clear that understanding the postoperative state is crucial to the wellbeing of a majority of patients, and yet several complicating factors impede our ability to develop experiments that lead to clear conclusions. Surgery in human patients has so many inherent variables that simple questions can be difficult to formulate, but surgery in animal models lacks clinical relevance. Our experiments are no exception, and yet this work has been designed to examine the fundamentals of the intestinal response to a surgical insult in normal animals as compared with those genetically predisposed to IBD.

These hypotheses and experiments explored the differences between animals that had undergone an intestinal transection/ reanastomosis, and those that had undergone a similar sham operation. Because the two groups were similar in all respects except

the intestinal incision and closure, the hypotheses related to the effect of the surgical insult itself could be tested. Unfortunately, this surgery differs in many crucial respects from surgery that would be done on humans. Preoperative bowel preparation and antibiotics, the possible use of intestinal stapling devices, dissolvable sutures, and perioperative parenteral nutritional support are only a few examples. For the purpose of these experiments, however, all of these factors, which could make the surgery further resemble surgery in humans, would also add additional variables. Thus, the possibility of producing results that are directly applicable to surgery in humans was sacrificed in favor of a model that allows the testing of fundamental hypotheses about the healing response in IBD.

SURGICAL RESULTS

Given their immunological deficit, it is not surprising that IL-10KO mice recover poorly from surgery as compared with WT, and it has been previously documented that IL-10KO mice recover poorly from sepsis(141). Interesting, was the fact that they had similar rates of operative-site complications, but a large proportion of animals became ill with an apparently intact surgical site. Unfortunately, determining the exact cause of death in these animals is impractical, but sepsis or systemic inflammatory response syndromes likely played a part in many of these deaths, given that both rates could be enhanced in animals with genetic immune defects. Our observation that operative sites seemed more fragile in IL-10KO animals highlights the close interaction between the immune system and healing response, and could be

further tested in experiments measuring post operative burst strengths. If our subjective observations represent a real weakness in postoperative healing strength in the IL-10KO animals, then that would be in keeping with the histological deficit we observed in fibroblast density at IL-10KO operative sites as compared with WT.

HISTOLOGY

As expected, wild type mice developed a pattern of histological injury characterized in the early phases predominantly by neutrophilic infiltration, and later by fibroblasts. While normal mice had a peak in collagen present around day 4, this was not seen in IL-10 knockout mice, perhaps explaining the qualitatively weaker anastomoses we observed in the early postoperative stage in IL-10KO mice. Fibrin deposition was not a feature of the histology observed within the first 16 days, but given that fibroblasts appeared late in that time period, fibrin likely increases as part of the later healing response.

The width of histological changes observed at anastomotic sites is an important result of our study, since it is the factor most clearly different between normal and IL-10KO mice. The wider area of injury and inflammatory cell infiltration seen in IL-10KO mice is compatible with the initial hypothesis of a deregulated immune response in the animals predisposed to IBD, but of interest, no changes compatible with IBD were seen in the IL-10KO animals following surgery.

INTESTINAL PERMEABILITY

Intestinal permeability was measured by an indirect method, bacterial translocation, and a direct method, mannitol flux. The finding that bacterial translocation was transiently increased postoperatively, and to a greater degree in operated than sham-operated animals was not supported by direct measurements of intestinal permeability at postoperative day 2, which showed decreased intestinal permeability in animals that had undergone surgery. This finding is in keeping with previous results in animal studies of small bowel resection, demonstrating that bacterial translocation is uncoupled from intestinal permeability following surgery(142). In view of these findings, it would seem that the increased growth of bacteria from abdominal viscera following intestinal surgery is more related to micro-leakage at the anastomotic site than to a true change in intestinal permeability. Thus, while bacterial translocation is not helpful in terms of the replication of our data by different testing strategies, it does serve as an interesting illustration that intestinal surgery in IBD patients can lead to increased exposure of the enteric immune system to luminal bacteria by means other than via transmural permeability.

On day 2 the mannitol flux results were statistically significant in terms of the decreased intestinal permeability seen in operated as compared to sham-operated animals, but permeability was also numerically decreased in the postoperative animals at every anatomical site of the small bowel in both IL-10KO and WT animals. This apparently widespread drop in intestinal permeability postoperatively

is in contrast to a localized histological pattern of insult, and, as will be discussed, a localized pattern of cytokine changes.

While these results are contrary to the hypothesis that permeability would be increased by intestinal surgery, the results have interesting implications, and suggest that intestinal permeability may be decreased as a protective response during surgical wound healing. The possibility that surgery results in a global decrease in intestinal permeability is supported by previous experiments, by O'Brien *et al*, who had also hypothesized that intestinal resection would result in increased intestinal permeability(143). They showed decreased intestinal permeability in Sprague-Dawley rats following intestinal resection, but attributed the decrease in permeability to an adaptive response to resection rather than to an effect of the surgery itself, an effect we can define more specifically in our experiments. O'Brien's studies demonstrated that permeability in RIEC-6 tissue monolayers could be decreased by postoperative serum from animals who had undergone a sham resection, similar to the operative procedure reported here, as well as from those who had undergone resection. This suggested a humoral factor responsible for the permeability drop. They found that permeability decreases induced by serum from animals that had undergone resection exceeded the drop induced by those that had undergone sham resection, thus their conclusion that the resection was the key factor. With the results obtained in these studies comparing transection/reanastomosis with a sham operation, it becomes apparent that the bowel incision is also a key factor that induces this widespread permeability drop.

This conclusion may help explain results that have previously been observed in humans. Koltun and colleagues found that abnormally elevated intestinal permeability in patients with ileocolonic Crohn's disease could be corrected by carrying out a resection of the diseased bowel(143). While this initially seems intuitive, it is surprising that removal of affected bowel could correct the entire permeability defect, given that many patients in Crohn's disease remission have abnormal pemeability(144), and intestinal permeability is even increased in relatives of patients with Crohn's(64, 145). These findings suggest that the permeability defect in Crohn's disease is at least partially independent of disease activity, and may involve the entire bowel. It is possible that the normalization of permeability in patients undergoing resection for Crohn's could be a result of healing responses to the surgical insult itself, rather than removal of the disease site.

Our day 7 results demonstrating equalization of permeability between sham and surgical animals in both IL-10KO and WT mice indicate that the postoperative permeability drop is a transient phenomenon. The fact that Il-10KO mice trended towards lower permeability than WT on postoperative day 2, and higher permeability on day 7, may suggest a relative permeability defect in the animals genetically prone to IBD at later stages of healing, but these experiments were not designed to explore this possibility further.

CYTOKINE MEASUREMENTS

Changes in cytokine levels surrounding a surgical site appear to be a phenomenon that is localized to within a few centimeters of the incision. These observations that quantitative cytokine measurements in the cecum were no different in sham than surgical animals, suggests that the large bowel is relatively uninvolved, even at the short distance of one centimeter from the operative site.

In WT mice, the peak of both TNF-α and TGF-β adjacent to the operative site indicates that both pro and anti-inflammatory cytokines play a role in the postoperative healing response. Qualitatively, they appear to be elevated within 1-2cm of the operative site, in contrast both to the very narrow histological margin of injury, and to the extensive region in which permeability is affected.

SYSTEMIC IMMUNE RESPONSIVENESS

Testing the hypothesis that surgery results in a proinflammatory state that is aberrantly stimulated in animals genetically predisposed to Crohn's disease depended first on defining immune responsiveness postoperatively in normal animals. However, rather than increased responsiveness postoperatively, it was observed that splenocytes from animals that had undergone surgery had dramatically decreased responsiveness to ConA, as compared with shams. Since these observations were taken seven days postoperatively, it would seem that by then, the predominantly inflammatory stage of the healing process is complete, and the inflammatory response

was being suppressed. That stimulation of lymphocytes by ConA as measured by IFN-γ production was suppressed postoperatively, but stimulation by anti-CD3 was not, is important, since the pathway by which ConA induces IFN-γ production is suppressible by IL-10(146). This leads to the critical difference in IL-10KO mice that the normal suppression of splenocyte responsiveness to ConA is lost. The subsequent results, demonstrating that postoperative WT mice react with a greater IL-10 response to a range of stimuli than sham-operated mice, adds evidence that IL-10 is key in the physiologic postoperative immune suppression.

The relatively suppressed immunological state that was observed in WT postoperative mice was surprising enough that it warranted replication of the experiments using different end points, and indeed a decreased responsiveness of WT splenic lymphocytes as measured by proliferation experiments was observed. In these experiments, a numerically decreased response to both ConA and anti-CD3 was observed for splenocytes of WT mice. Once again, this normal suppression of systemic immune responsiveness was lost in IL-10KO mice, where surgical animals had slightly higher (but similar) responsiveness than their sham-operated counterparts.

While this series of experiments was contrary to the hypothesis, the results do provide key clues towards possible pathogenic factors in postoperative Crohn's recurrence. Rather than, as was expected, a heightened immune response that is abnormally brisk in IL-10KO mice, a normal suppression of the immune response postoperatively was

observed that the animals genetically prone to IBD could not accomplish. Since some of this normal suppressive mechanism is likely IL-10 mediated, it may come as no surprise that this would be the nature of the defect in IL-10KO mice. As discussed in the introduction of this manuscript, however, the pathways through which IL-10KO mice develop and sustain IBD involves many pathways downstream of IL-10 that can resemble human IBD. For this reason, the replication of these experiments in different animal models of IBD would be interesting. Although the TGF-β knockout mouse might provide some of the most relevant results, the key role played by TGF-β in healing, and the challenging surgical mortality in these experiments, suggests that this model might have unmanageable levels of postoperative mortality.

FLOW CYTOMETRY

Flow cytometry experiments were carried out to explore reasons for the dramatic decrease in splenocyte responsiveness to ConA. It was hoped that these would determine whether this decrease might be due predominantly to a change in splenic cell population (ie. lack of T-cells), or whether the T-cells were truly hyporesponsive. The slight trend towards a decrease in relative numbers of T-cells in postsurgical WT mice as compared with sham-operated may contribute to the decrease in proliferation seen, but is inadequate to explain the dramatic decrease in IFN-γ production in response to ConA.

CHAPTER 6

CONCLUSIONS

Following intestinal surgery, the normal response to injury involves an early infiltration of neutrophils, followed later by fibroblasts. Collagen deposition is increased in the first four postoperative days. Intestinal permeability is decreased, likely over the entire length of the small intestine, perhaps as a protective response against bacterial translocation. Cytokines are produced locally at the wound site that have an equivocal effect on IBD pathogenesis, since they include both IBD-stimulating and suppressant molecules. By seven days postoperatively, systemic immune responsiveness is being suppressed, a phenomenon that likely involves production of IL-10. Intestinal permeability changes are no longer evident at this time.

In IL-10KO mice, animals genetically predisposed to IBD, the normal postoperative decrease in intestinal permeability is retained. Abnormalities identified in these experiments that resemble pathophysiologic mechanisms of inflammatory bowel disease include a widened histological zone of injury and a lack of normal IL-10 dependent systemic immune downregulation 1 week postoperatively.

Replication of these results in other animal models of Crohn's disease would suggest that they can be generalized to common pathways of IBD expression. Novel therapeutic approaches to the management of postoperative Crohn's disease should

focus on mechanisms identified as abnormal in the injury response of genetically susceptible hosts.

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Table 1: Evidence for interventions to prevent postoperative Crohn's recurrence

TREATMENT	LEVEL OF EVIDENCE	RESULTS	NOTES
Blood transfusion	Retrospective	No effect	Individual papers suggest effect; not supported by meta-analysis(98)
Sulfasalazine	3 randomized prospective trials of Sulfasalazine versus placebo(99-101)	Overall improved recurrence rates at one year	Meta-analysis supports efficacy at 1-year(102). Studies with 3 years of follow-up: no benefit
Mesalamine	6 RCT's* (103, 104, 106-109)	Mixed results; heterogeneous studies	Meta-analysis suggests 13% improvement in 1-year recurrence rate by mesalamine(110)
Nitroimidazole antibiotics	2 RCT's 1) 60 patients randomized to metronidazole or placebo(114) 2) 80 patients randomized to ornidazole or placebo	1- year recurrence 4% for metronidazole, 25% for placebo 1-year recurrence 7.9% for ornidazole, 37.5% for placebo	These trials support drug effect, but appropriate dose and duration remain questionable. Side effects are an issue.
6-MP/AZA	3 RCT's** 1) NCCDS: 21 Patients AZA vs. Placebo(99) 2) 131 patients randomized to 6-MP, mesalamine or placebo(106) 3) 142 patients; AZA versus mesalamine(121)	AZA-Improved outcome rankings 2-year recurrence 50% for 6-MP versus 77% for placebo 2-year recurrence rates 28% for mesalamine, 17% for 6-MP (p=ns)	Study #2 showed no significant difference in recurrence rates, but survival analysis was significantly improved on 6-MP (low dose, at 50mg/day). Overall interpretation of results is controversial in the literature, but many authors recommend 6-MP/AZA for high risk patients.
Steroids	Prednisone: NCCDS data on 12 patients(99) Budesonide: 1 RCT of 129 patients(113)	Improved outcome ranking at 12 months 1 year endoscopic recurrence 63% for treatment and placebo groups	The use of steroids is not recommended for prophylactic therapy of Crohn's disease in any setting.
Probiotics	2 RCT's: 1) Lactobacillus rhamnosus GG in 45 patients(122) 2)VSL#3 (following rifaximin) in 40 patients(123)	-No difference in clinical or endoscopic recurrence rates -Endoscopic recurrence 20% for VSL#3 vs. 40% placebo (p=0.05)	Available RCT's are inadequate to support the use of any probiotic for post-op Crohn's maintenance. The VSL#3 RCT remains in abstract form.

*One additional RCT was published in abstract form only in 1991(147)

**One additional RCT was published in abstract form only in 1996(148)

Table 2: Randomized placebo-controlled trials assessing 5-ASA and sulfasalazine in the maintenance of surgical remission of Crohn's disease*

Study Drug (dose)	N	Recurrence definition	Treatment duration	Recurrence rate	Comments
Sulfasalazine (weight-based up to 5g/day) (99)	28	Clinical	12 months	No difference in outcome rankings	p=0.12
Sulfasalazine(3g/d) (100)	66	Clinical	12 months	Tx: 13% Placebo: 26%	p=ns
Sulfasalazine(3g/day) (101)	232	Radiology, endoscopy or surgery	3 years	1 year: -Tx 16% -Placebo 28% 3 years: -Tx 38% -Placebo 48%	1 year: p<0.01 3 years: p=0.09
Pentasa(4g/d) (103)	318	Clinical	18 months	Tx: 25% Placebo: 31%	p=0.10
Rowasa/Salofalk (3g/day)(104)	163	Clinical	72 months, variable F/up	Tx: 31% Placebo 41%	p=0.03
Pentasa(3g/day) (107)	66	Clinical	11 months	Tx: 10% Placebo: 23%	p=ns
Pentasa(3g/day) (108)	87	Clinical, endoscopic	12 months	Improved endoscopic scores and CDAI in treated group	Recurrence rates analyzed secondarily were not different
Claversal(3g/day) (109)	126	Endoscopic	3 months	Tx: 50% Placebo: 63%	p=0.16
Pentasa(3g/day) (106)	131	Clinical, endoscopic or radiologic	2 years	Tx: 58% Placebo: 77%	Trial included a 6-MP arm. Mesalamine was not statistically different from 6-MP or placebo

* One additional study was published in abstract form only in 1991(147)

Table 3 : Polymerase Chain Reaction Primer Sequences

Primer name	Sequence
IFN-γ forward primer	5' TAC TGC CAC GGC ACA GTC ATT GAA 3'
IFN-γ reverse primer	5' GCA GCG ACT CCT TTT CCG CTT CCT 3'
TNF-α forward primer	5' ATG AGC ACA GAA AGC ATG ATC 3'
TNF-α reverse primer	5' TAC AGG CTT GTC ACT CGA ATT 3'
TGF-β forward primer	5' CGG GGC GAC GGG CAC CAT CCA TGA 3'
TGF-β reverse primer	5' CTG CTC CAC CTT GGG CTT GCG ACC CAC 3'
Actin forward primer	5' CCT GTG GCA TCC ATG AAA CT 3'
Actin reverse primer	5' GTG CTA GGA GCC AGA GCA GT 3'

Table 4: Postoperative mortality rates.

Mouse genotype	Procedure	Postoperative mortality
WT	Sham	0%
WT	Surgery	10%
IL-10KO	Sham	15%
IL-10KO	Surgery	47%

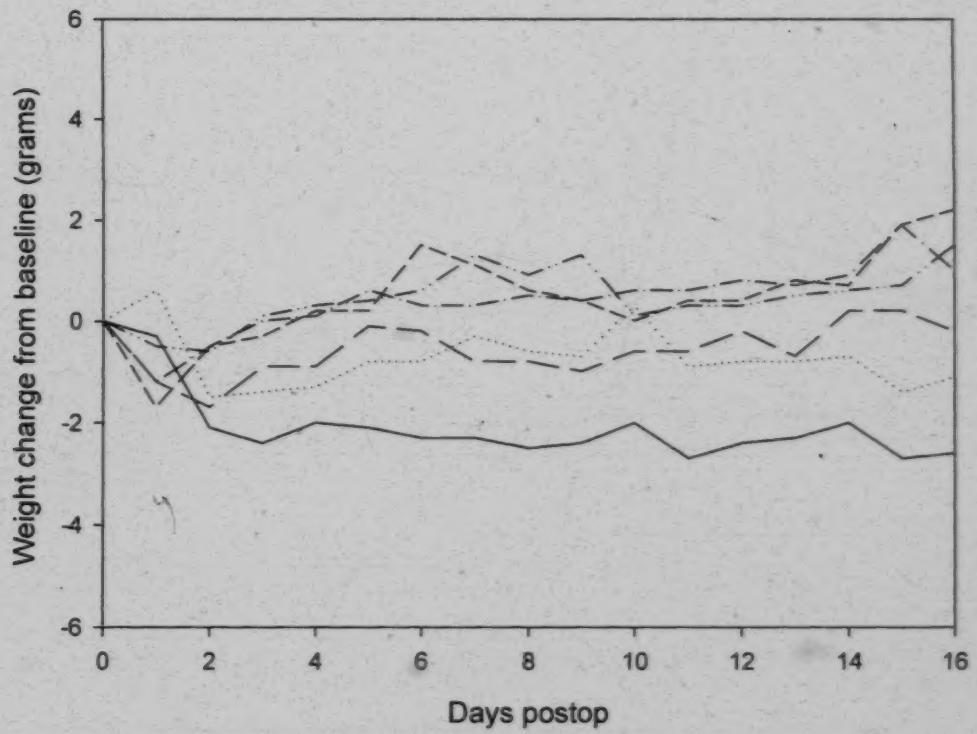

Figure 1: Growth curves of WT sham mice (n=6). Lines represent weights for individual mice.

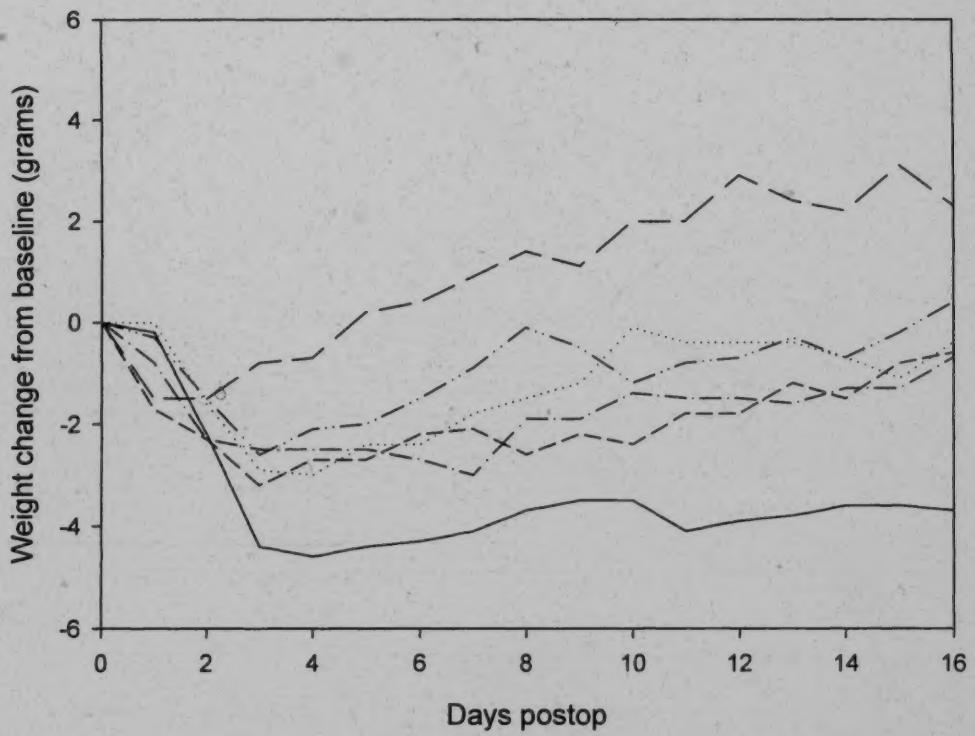

Figure 2: Growth curves of WT surgical mice (n=6). Lines represent weights for individual mice.

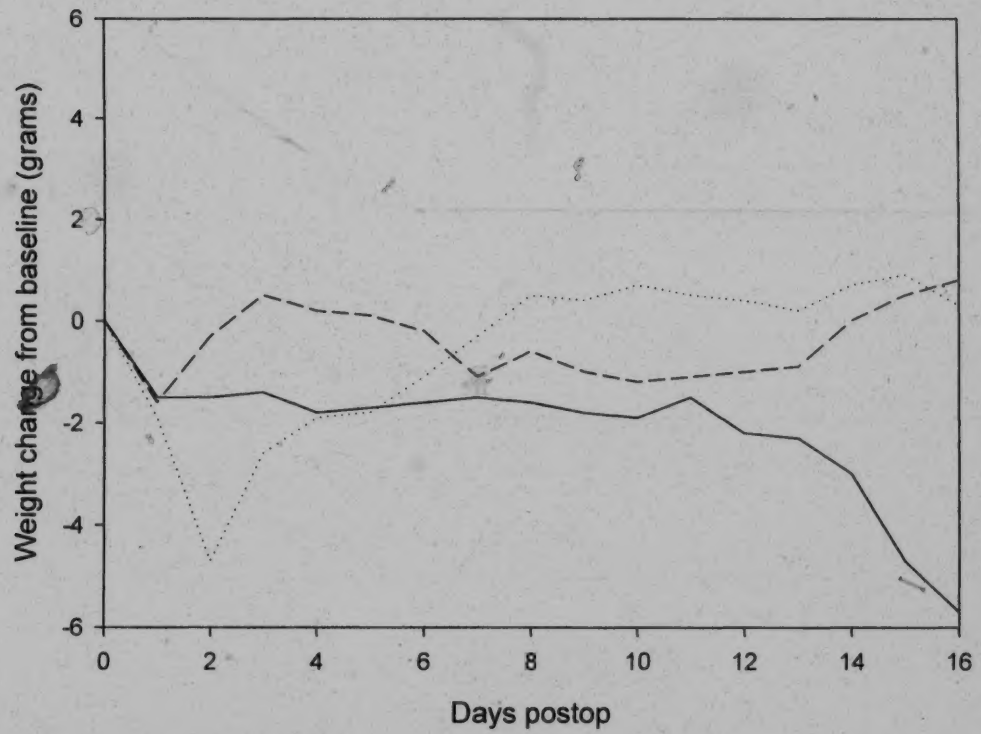

Figure 3: Growth curves of IL-10KO sham mice (n=3). Lines represent weights for individual mice.

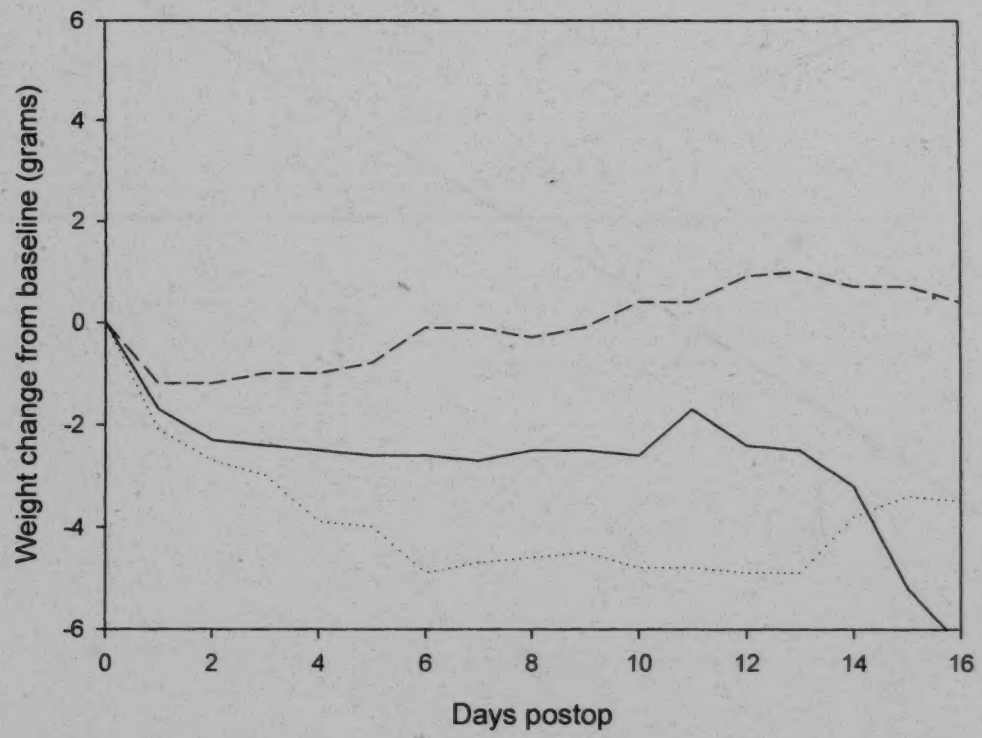

Figure 4: Growth curves of IL-10KO surgery mice (n=3). Lines represent weights for individual mice.

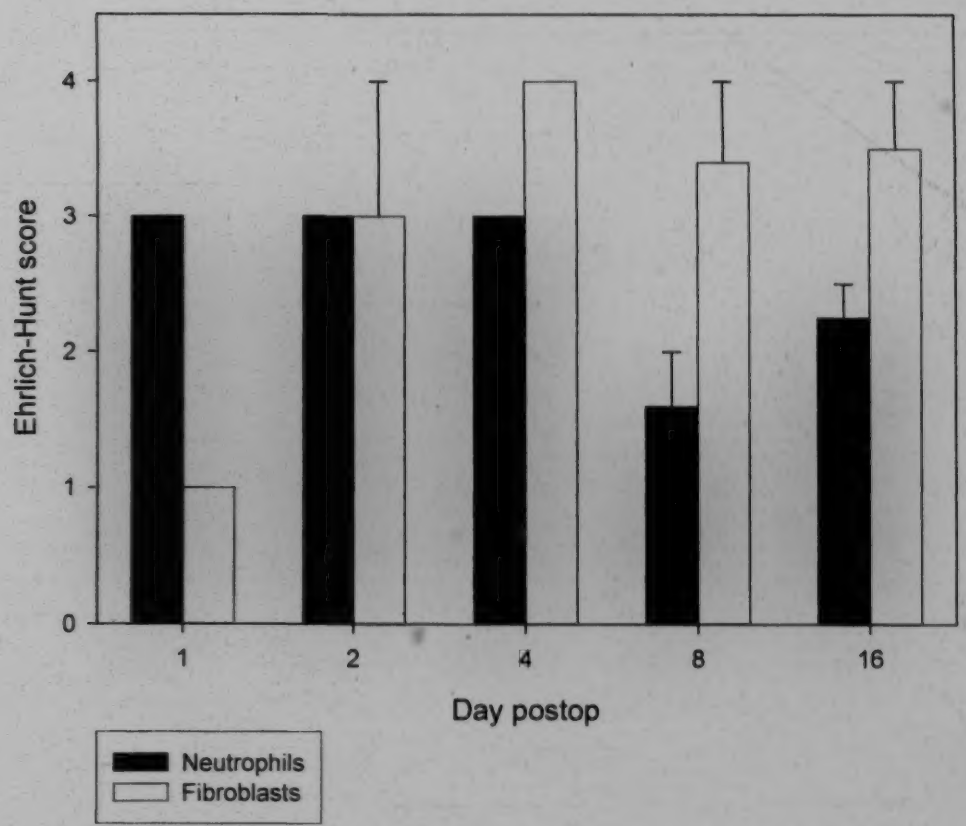

Figure 5: Anastomotic site histological changes with time in WT surgical mice. Zero = no injury, 4 = maximal injury (n=6 at each timepoint).

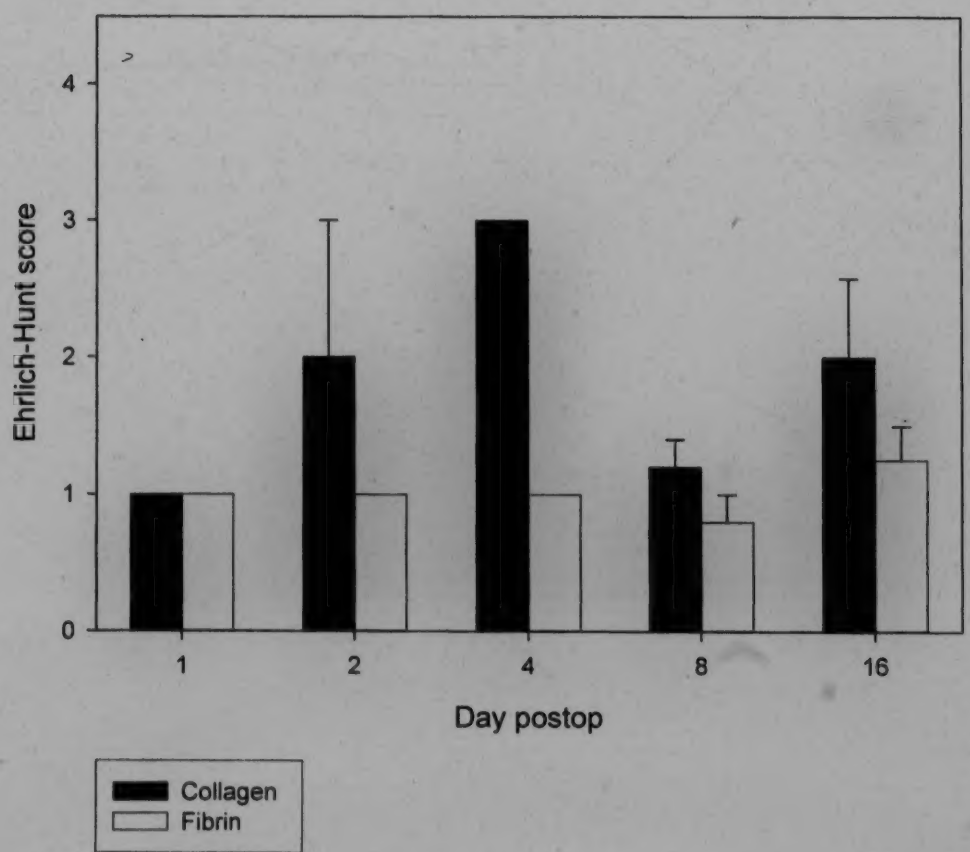

Figure 6: Structural histologic parameters in WT surgical mice. Zero = no injury, 4 = maximal injury (n=6 at each timepoint).

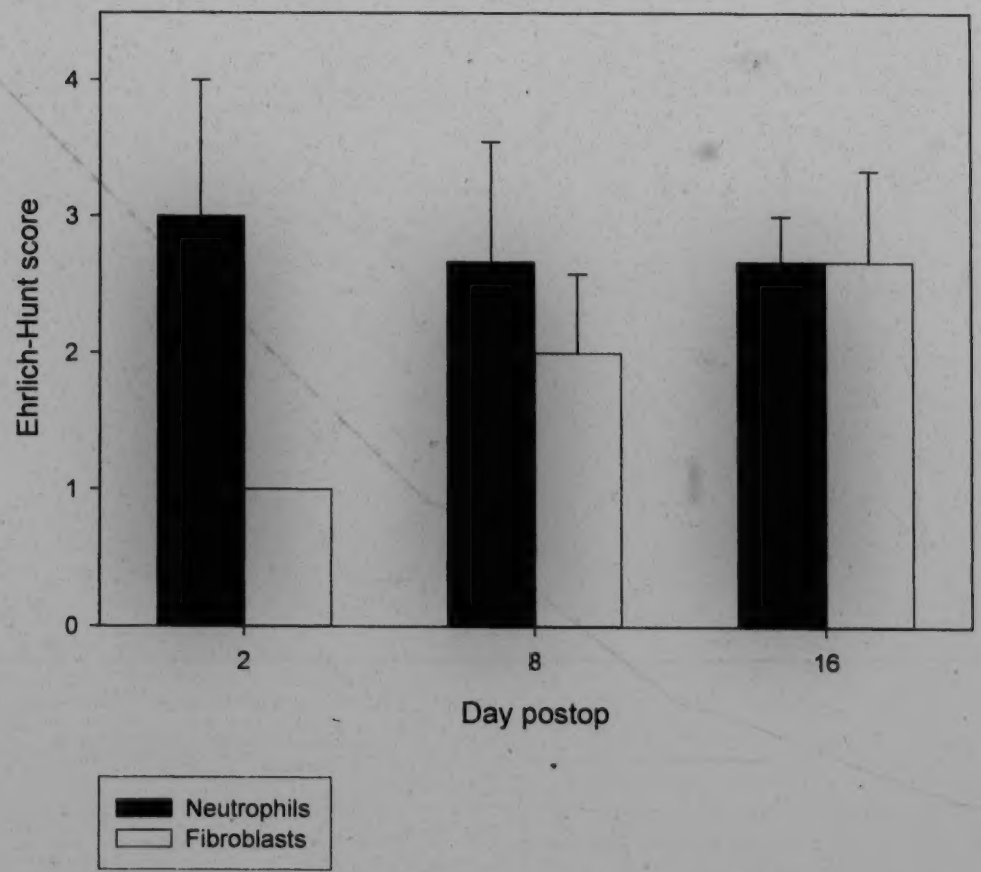

Figure 7: Anastomotic site histological changes with time in IL-10KO surgical mice. Zero = no injury, 4 = maximal injury (n=3 at each timepoint).

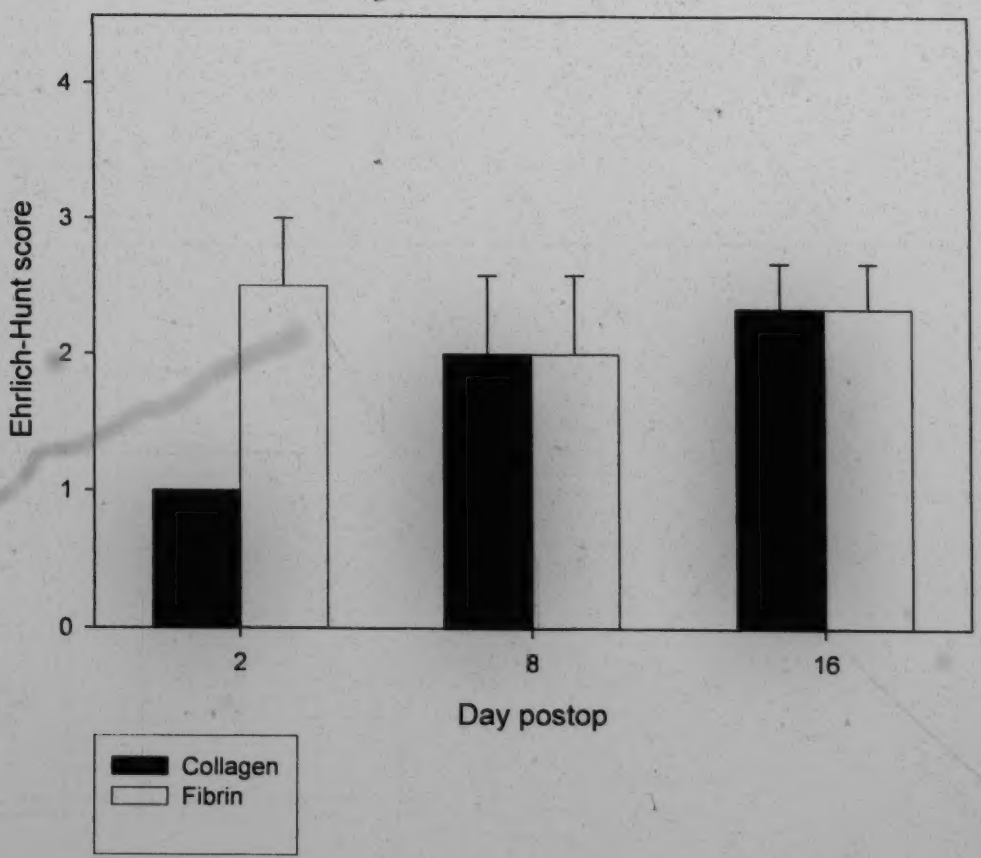

Figure 8: Structural histologic parameters in IL-10KO surgical mice. Zero = no injury, 4 = maximal injury (n=3 at each timepoint).

Figure 9: Histological photograph of normal mouse small bowel. Villous architecture and epithelial continuity are present.

Figure 10: Histological photograph of WT surgical mouse small bowel on postoperative day 2. Epithelial discontinuity and neutrophilic infiltrate are present.

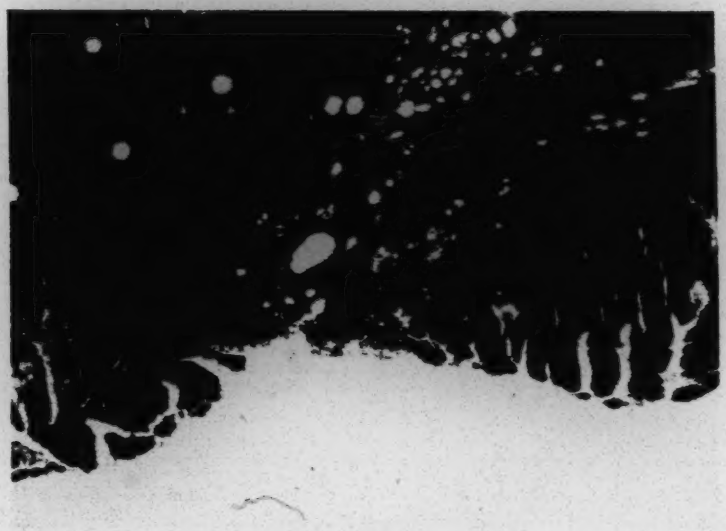

Figure 11: Histological photograph of WT surgical mouse small bowel on day 16 postoperatively. Inflammatory infiltrate is less dramatic and epithelial continuity is being restored.

Figure 12: Histology from an IL-10KO surgical mouse on postoperative day 2. This again shows intense neutrophilic infiltrate. Suture material can be seen in this photograph (arrow).

Figure 13: Histology at higher magnification from an IL-10KO surgical mouse on postoperative day 2.
The infiltrate is predominantly PMN's.

Figure 14: Histology from day 8 in an IL-10KO surgical mouse. Increasing fibroblasts and less PMN's are seen.

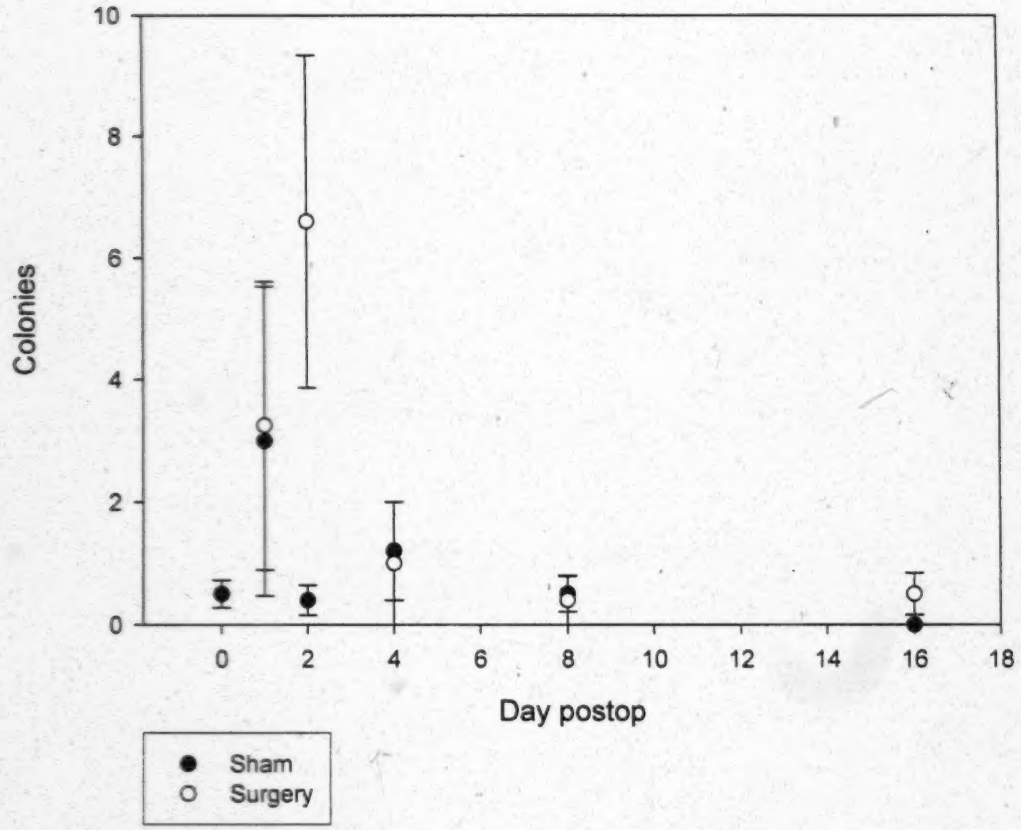

Figure 15: Bacterial translocation experiments in WT mice. Number of bacterial colony forming units (Colonies) were obtained from splenic cultures (n=6 at each timepoint).

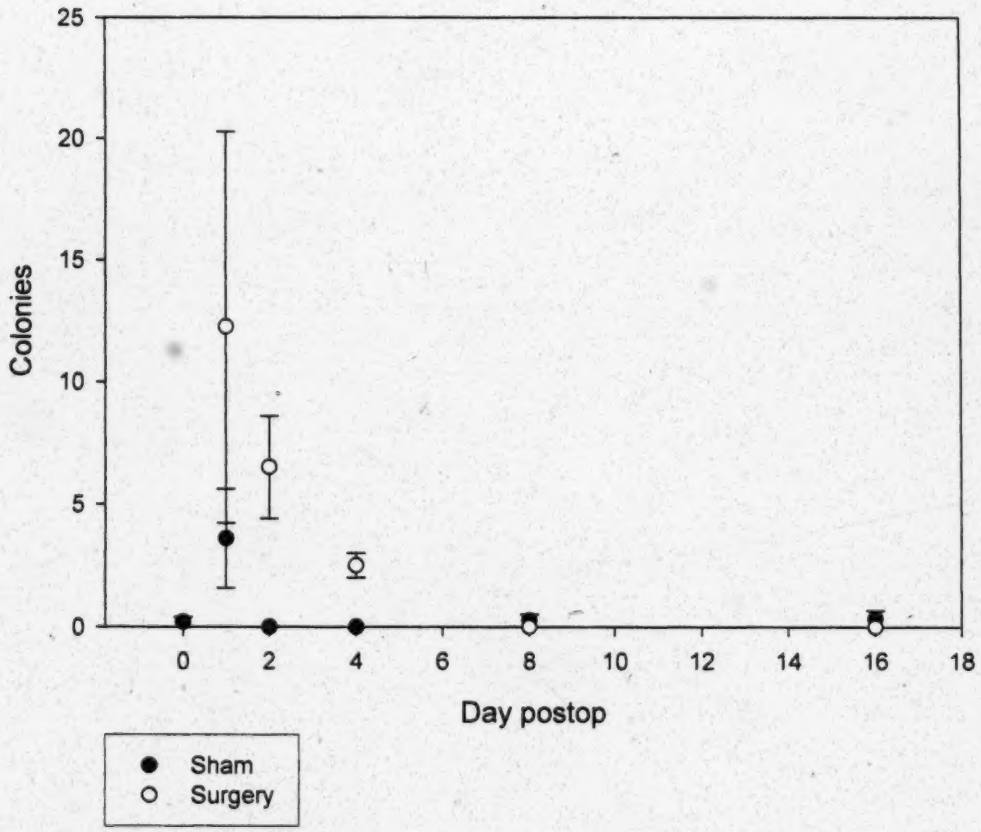

Figure 16: Bacterial translocation experiments in WT mice. Number of bacterial colony forming units (Colonies) were obtained from liver cultures (n=6 at each timepoint).

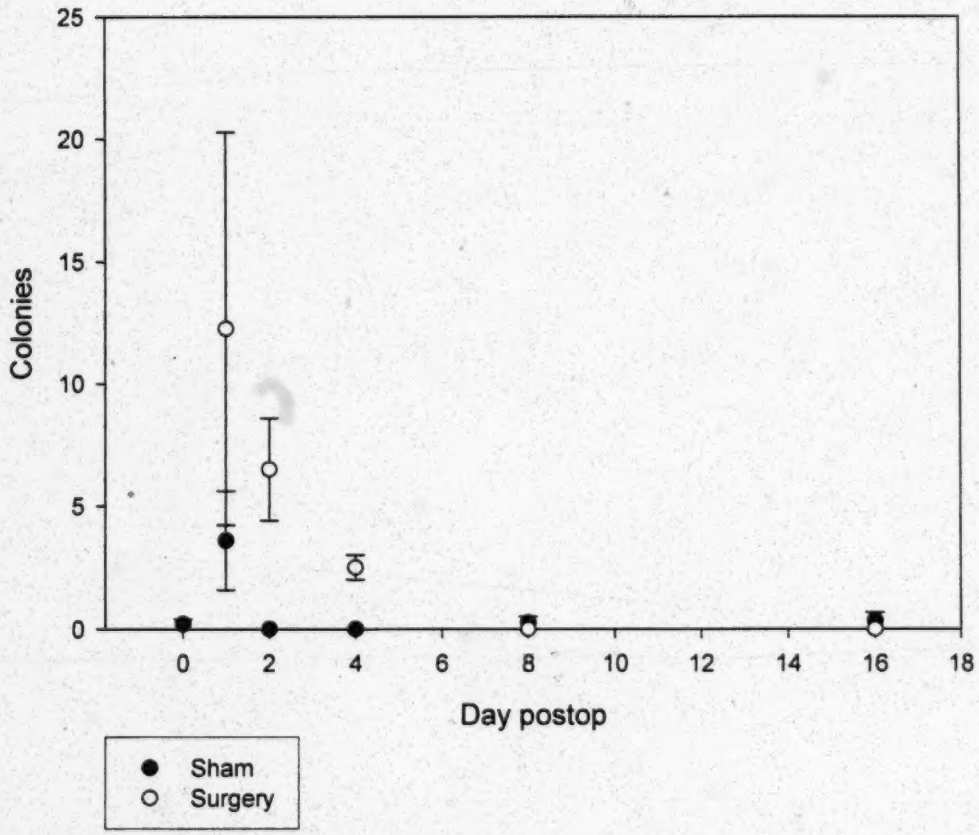

Figure 17: Bacterial translocation experiments in IL-10KO mice. Number of bacterial colony forming units (Colonies) were obtained from liver cultures (n=3 at each timepoint).

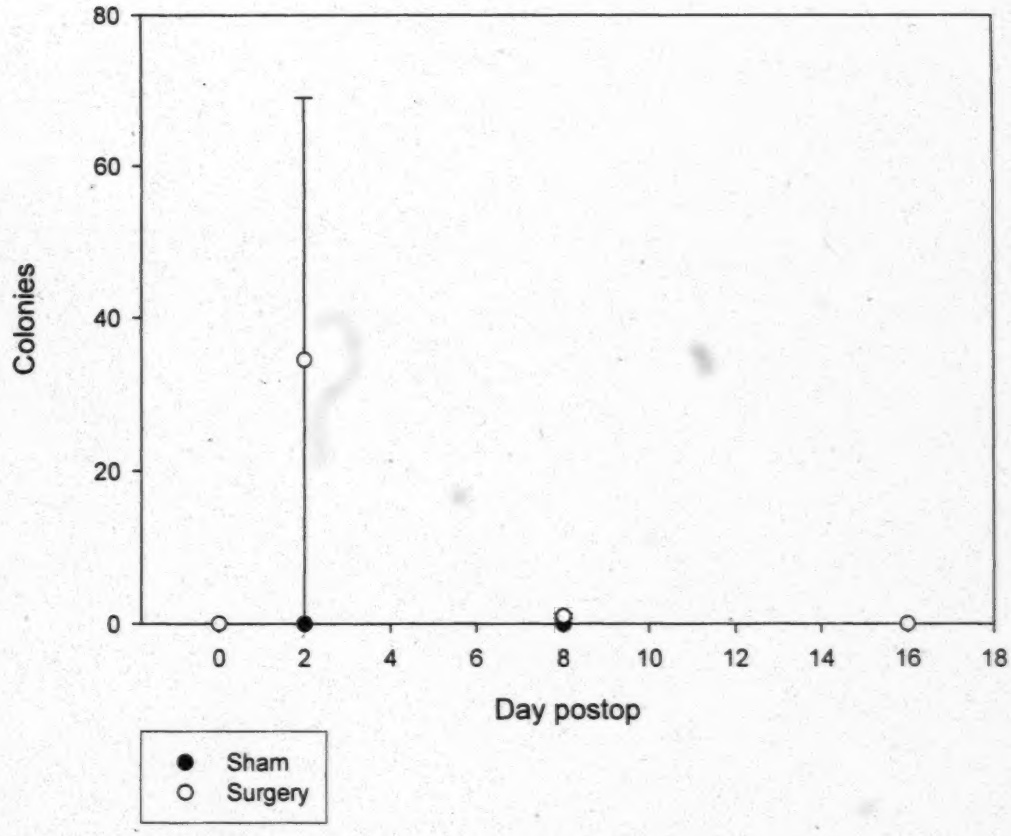

Figure 18: Bacterial translocation experiments in IL-10KO mice. Number of bacterial colony forming units (Colonies) were obtained from splenic cultures (n=3 at each timepoint).

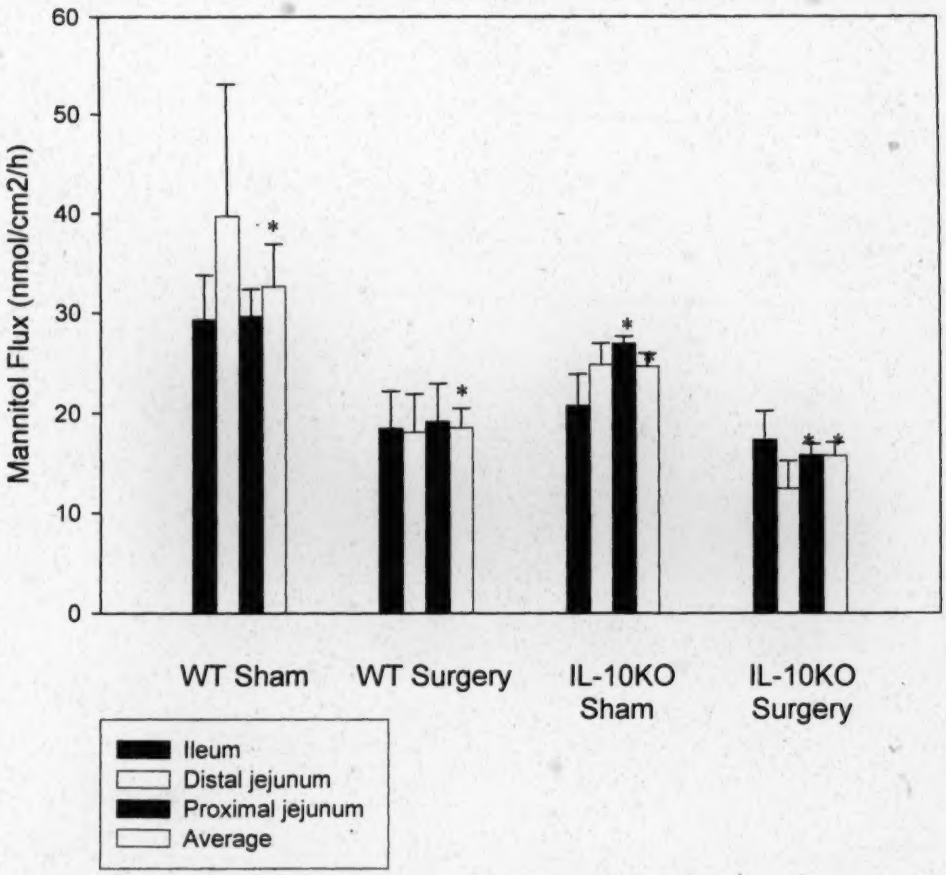

Figure 19: Intestinal permeability in WT and IL-10KO mice on postoperative day 2 (n=4 in each group). Intestinal permeability is expressed as transmural mannitol flux (nmol/cm2/h).

* In WT mice, p=0.003 for comparison of average permeabilities in sham versus surgical mice. Comparisons at individual intestinal sites were not significant (p>0.05).

* In IL-10KO mice, p=0.001 for comparison of average permeabilities in sham versus surgical mice, and p=0.014 for comparison of permeabilities at the proximal jejunal site. Comparisons at the ileal and distal jejunal sites were not significant (p>0.05).

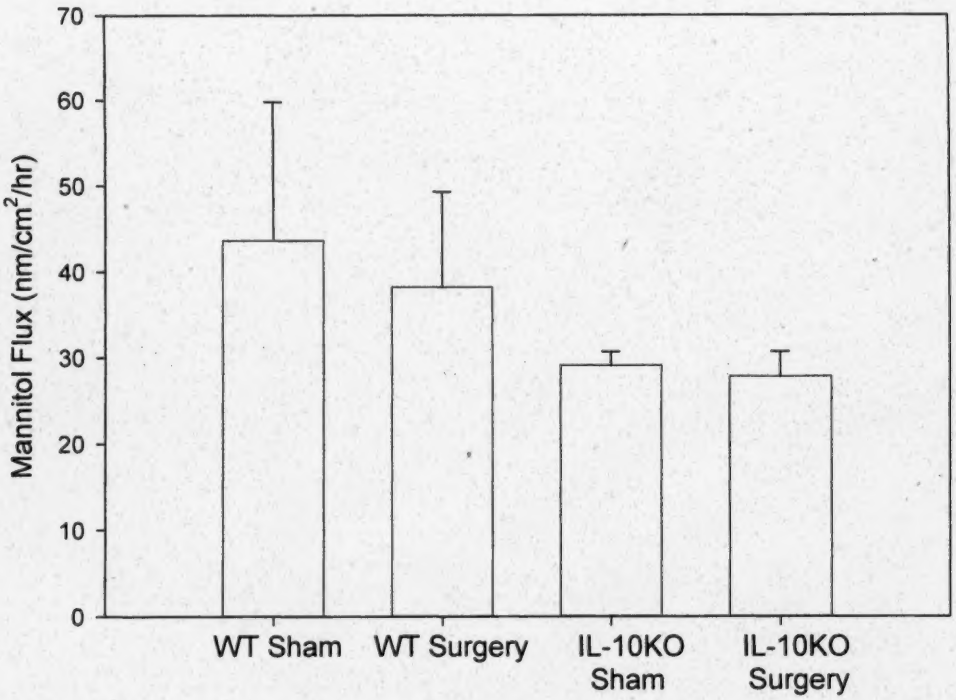

Figure 20: Intestinal permeability on postoperative day 7 (n=6 samples used for each group). Intestinal permeability is expressed as transmural mannitol flux (nmol/cm2/h). Comparisons were not significant ($p>0.05$) for each sham vs surgery comparison.

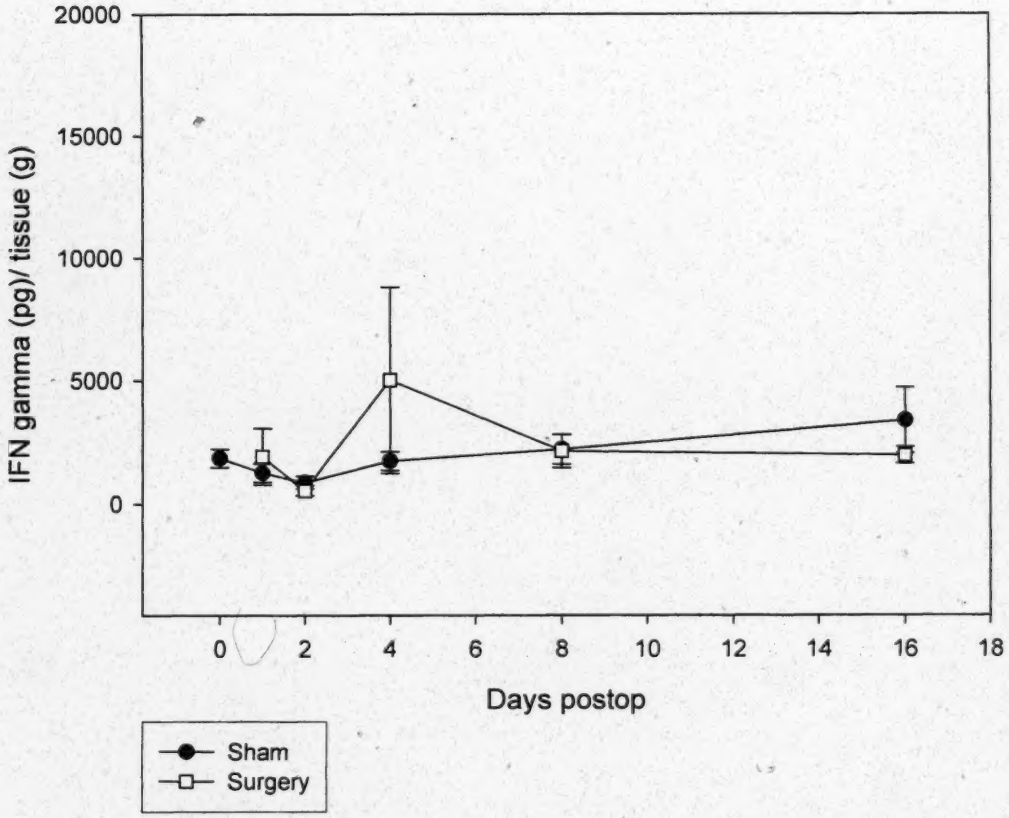

Figure 21: Postoperative cecal mucosal IFN-γ in WT mice (n=6 animals at each data point).

*p-values are > 0.05 for comparisons between surgery and sham-operated mice at all time points

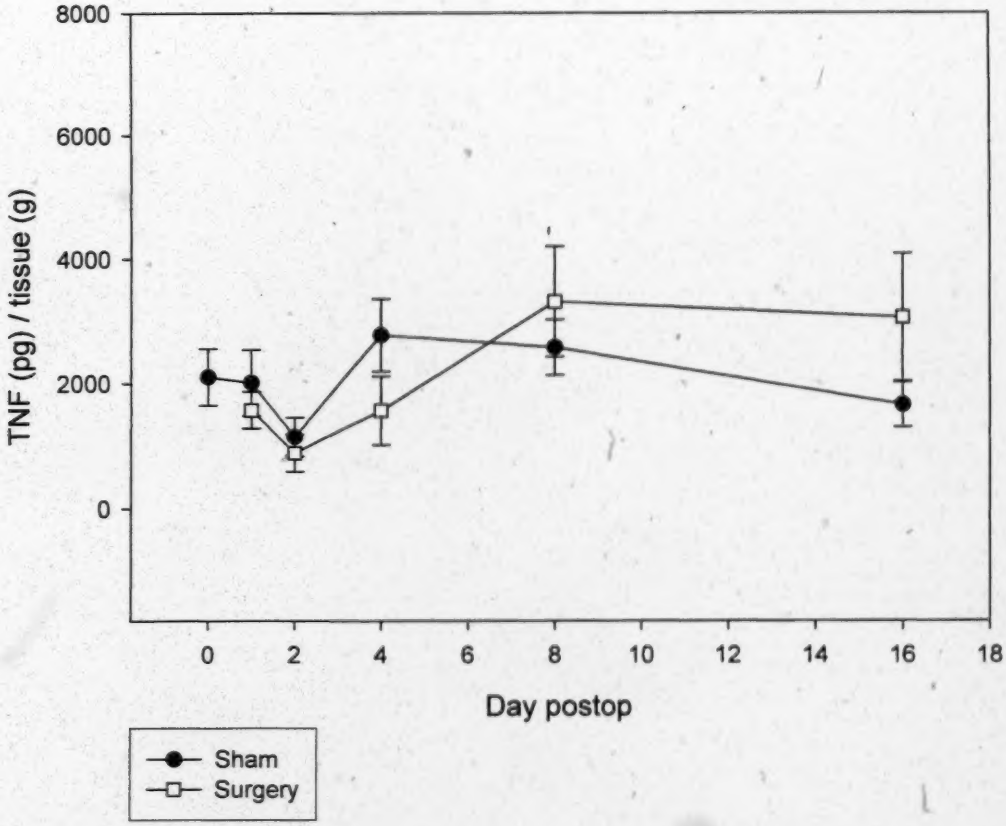

Figure 22: Postoperative cecal mucosal TNF-α in WT mice (n=6 animals at each data point).

*p-values are >0.05 for comparisons between surgery and sham-operated mice at all time points

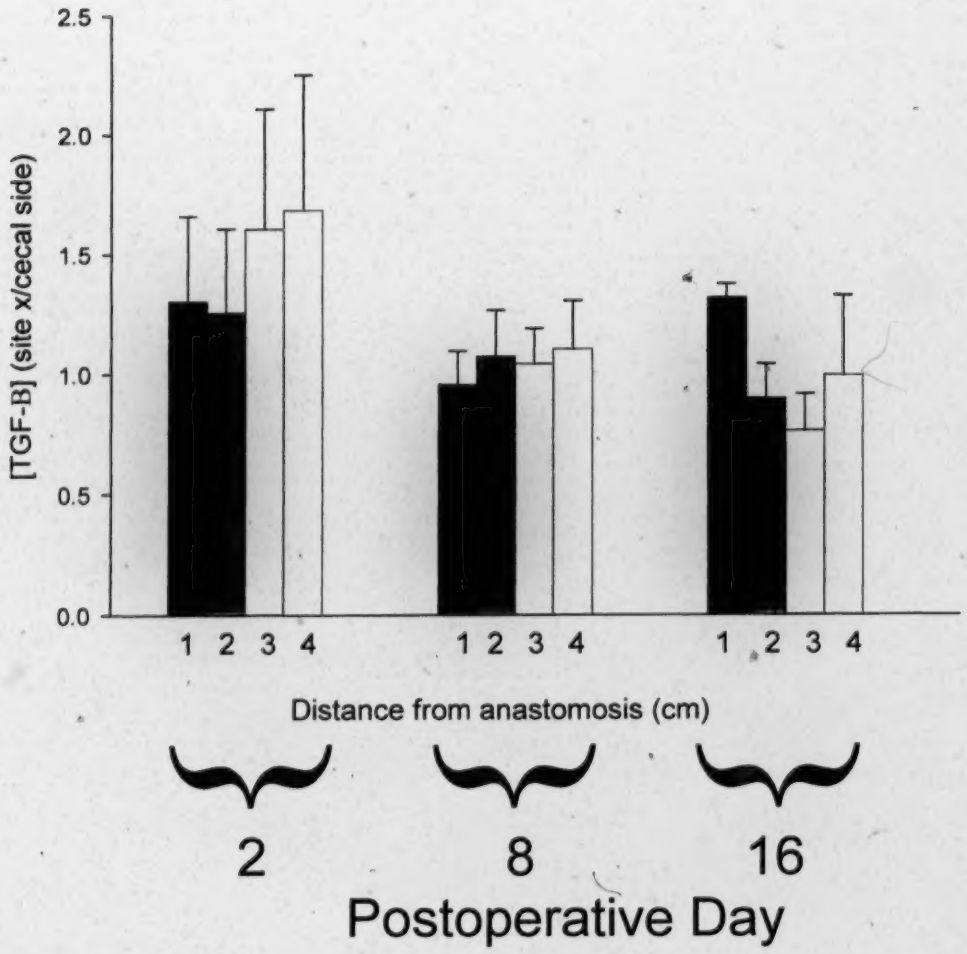

Figure 23: Mucosal TGF-β mRNA proportional expression expressed as distance from anastomotic site on various postoperative days in sham animals (n=6 animals for each data point).

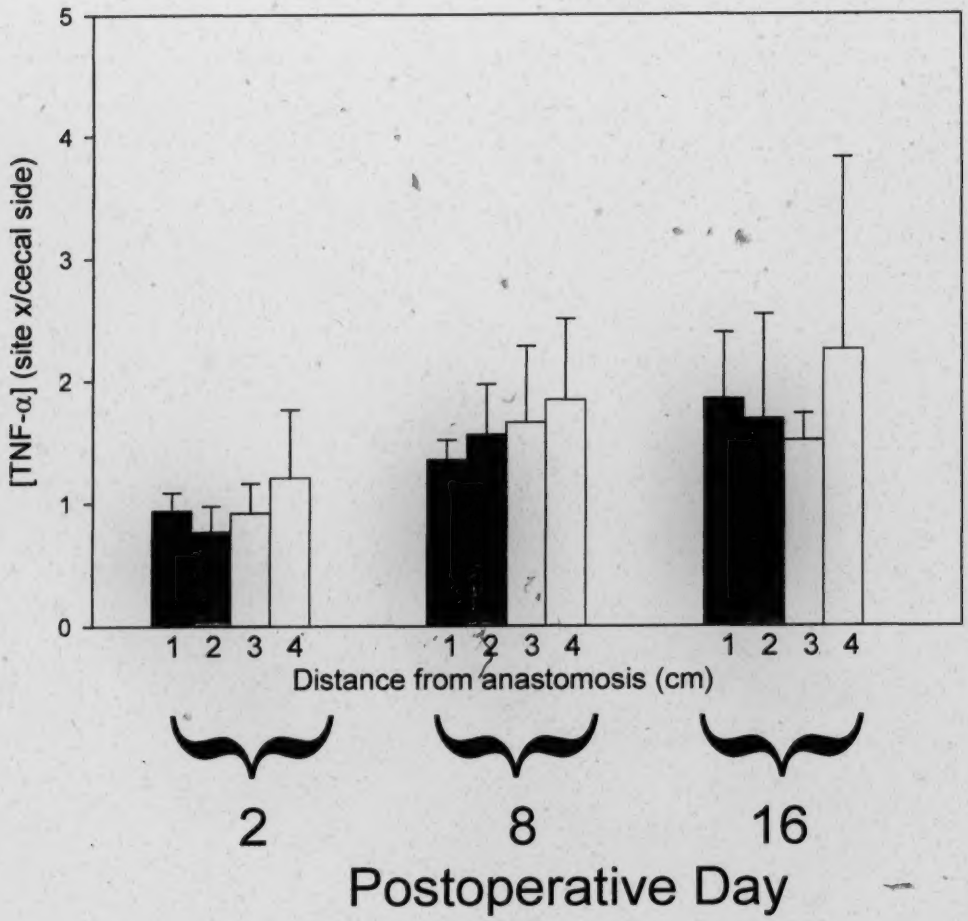

Figure 24: Mucosal TNF-α mRNA proportional expression expressed as distance from anastomotic site on various postoperative days in sham animals (n=6 animals for each data point).

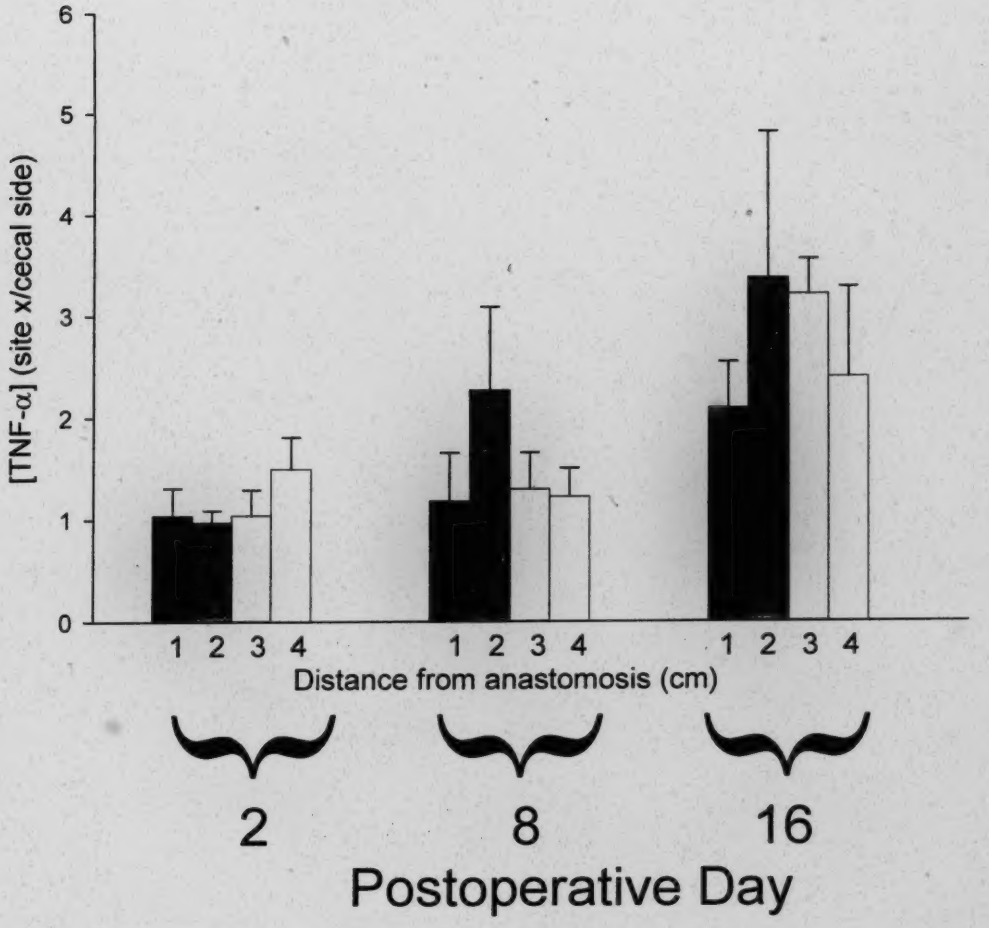

Figure 25: Mucosal IFN-γ mRNA proportional expression expressed as distance from anastomotic site on various postoperative days in sham animals (n=6 animals for each data point).

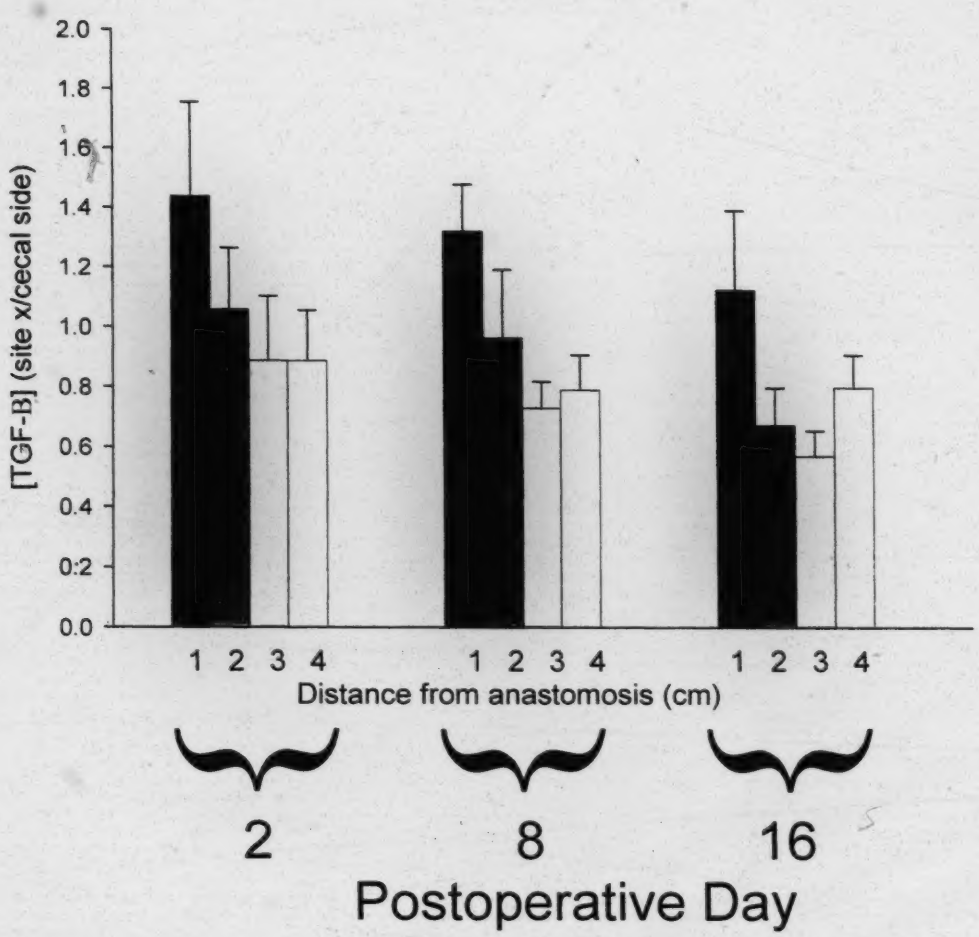

Figure 26: Mucosal TGF-β mRNA proportional expression expressed as distance from anastomotic site on various postoperative days in surgical animals (n=6 animals for each data point).

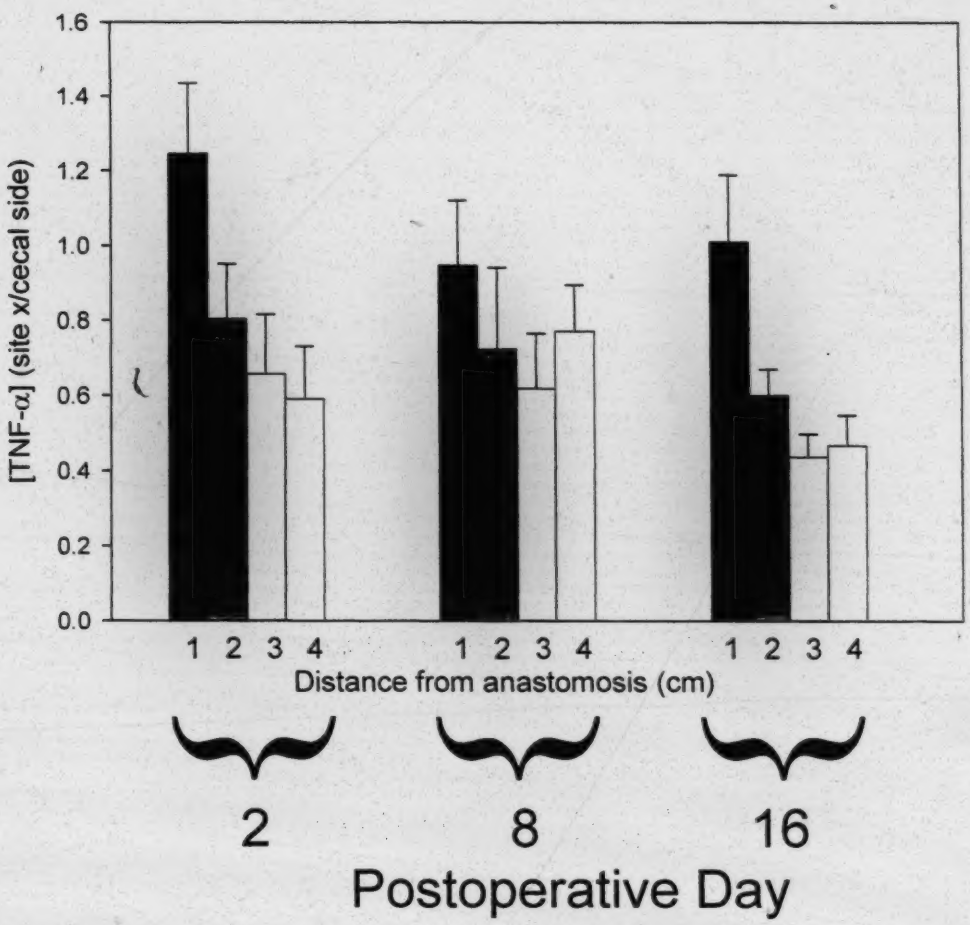

Figure 27: Mucosal TNF-α mRNA proportional expression expressed as distance from anastomotic site on various postoperative days in sham animals (n=6 animals for each data point).

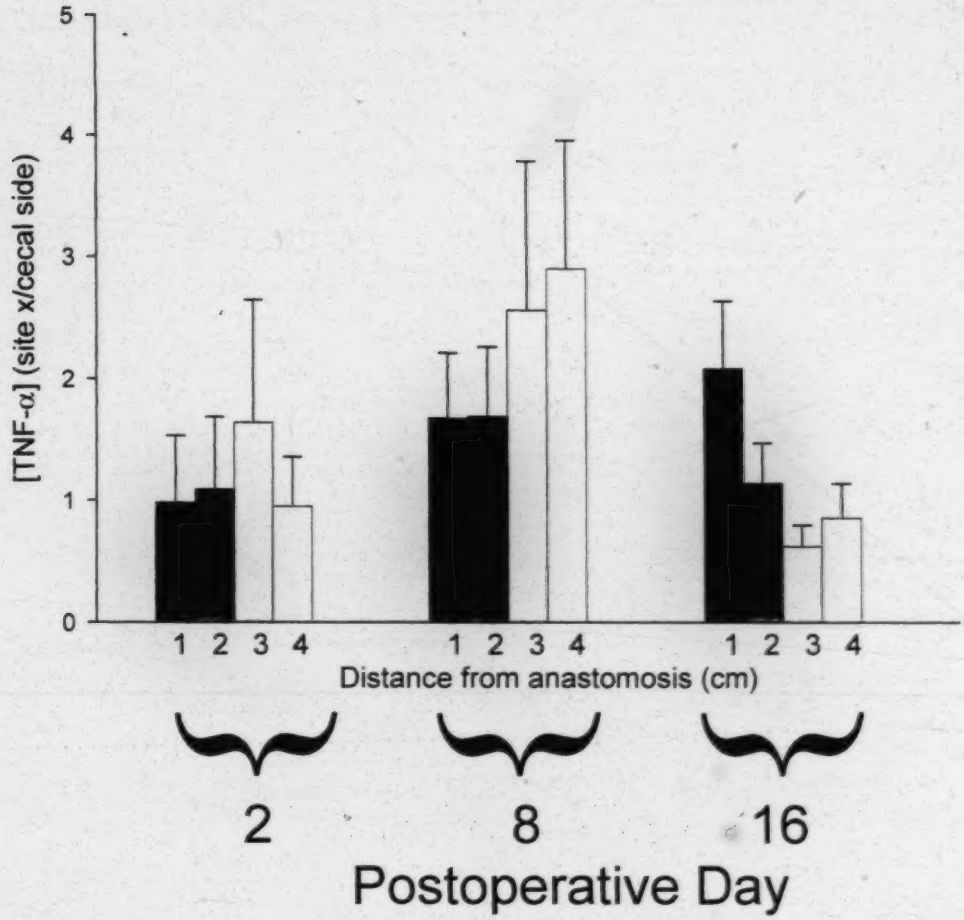

Figure 28: Mucosal IFN-γ mRNA proportional expression expressed as distance from anastomotic site on various postoperative days in surgical animals (n=6 animals for each data point).

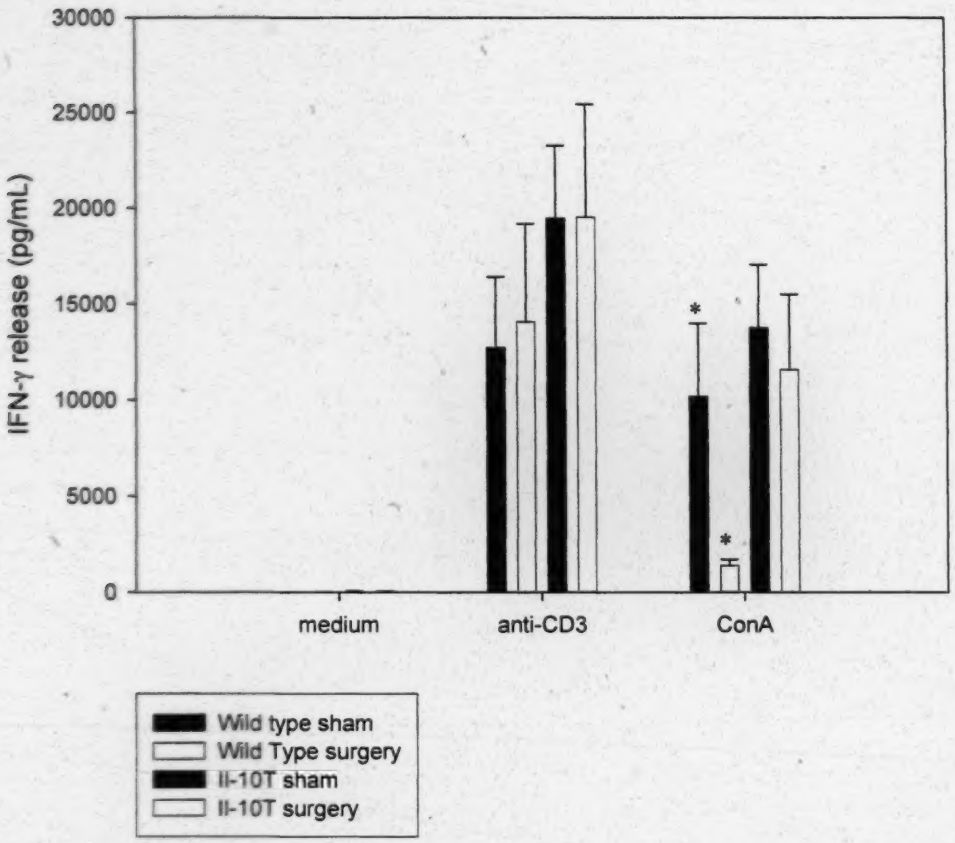

Figure 29: Splenic lymphocyte responsiveness, as determined by IFNγ release, to anti-CD3 and Con A in WT and IL-10KO animals who underwent sham or surgical procedures (n=7 in each group).

*p=.003 for comparison between sham and surgical animals stimulated with ConA. Other sham-surgical comparisons are not statistically significant (p>0.05).

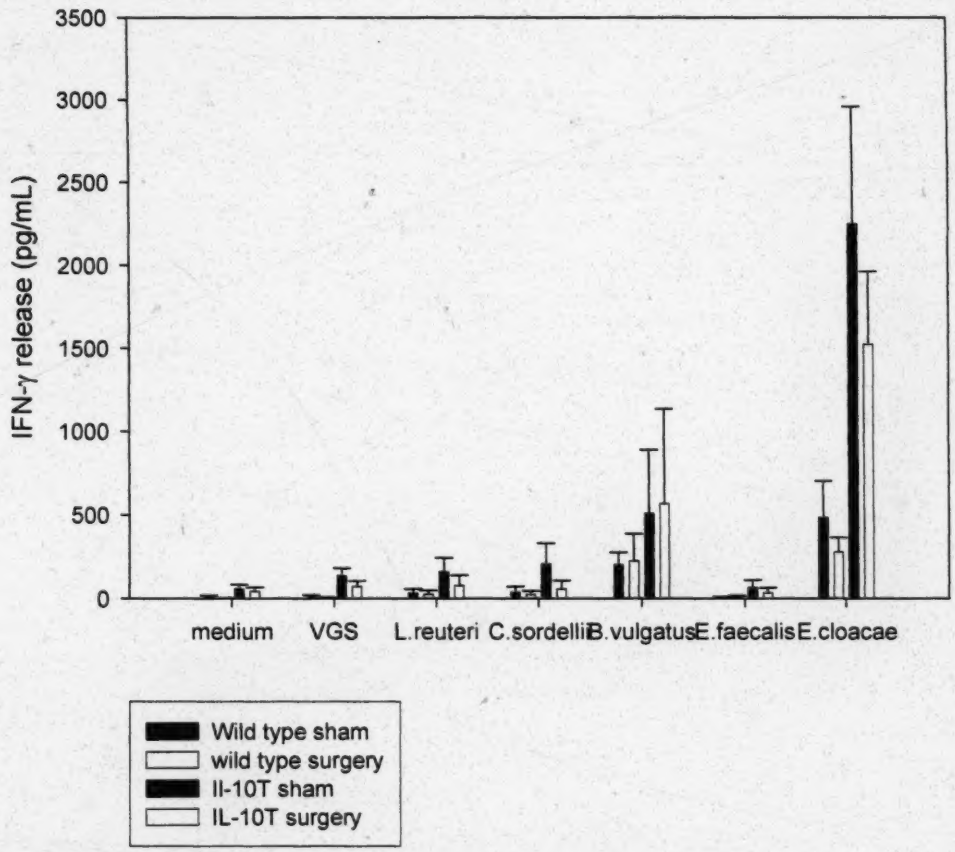

Figure 30: Splenic lymphocyte response, as determined by IFNγ release, to stimulation by bacterial sonicates (n=4 in each group). All comparisons between surgical and sham animals are not statistically significant ($p>0.05$).

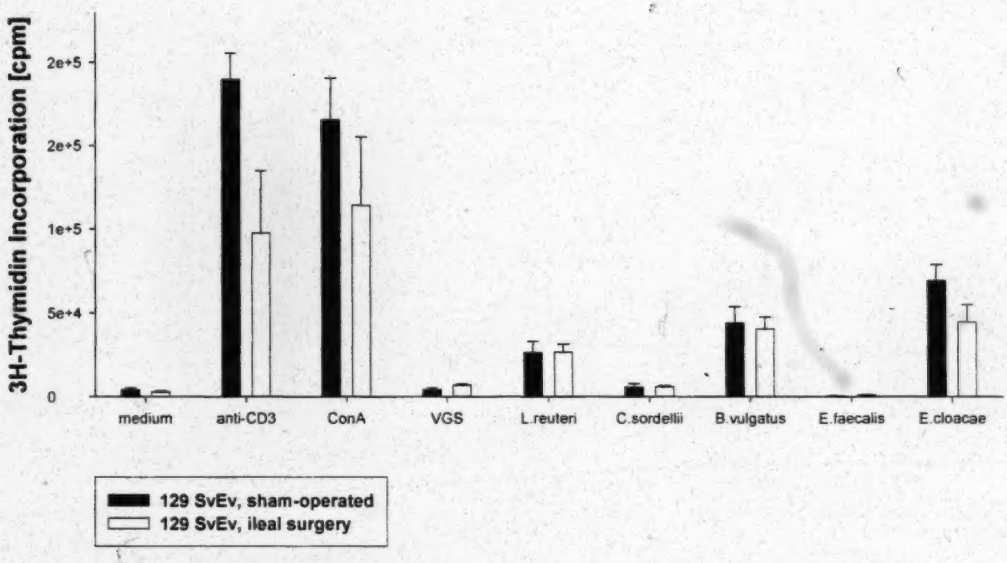

Figure 31: WT Splenic lymphocyte responsiveness to chemical stimulants and bacterial sonicates as measured by thymidine incorporation (cell proliferation) (n=4 for each group). All comparisons between sham and surgical animals are not significant ($p>0.05$).

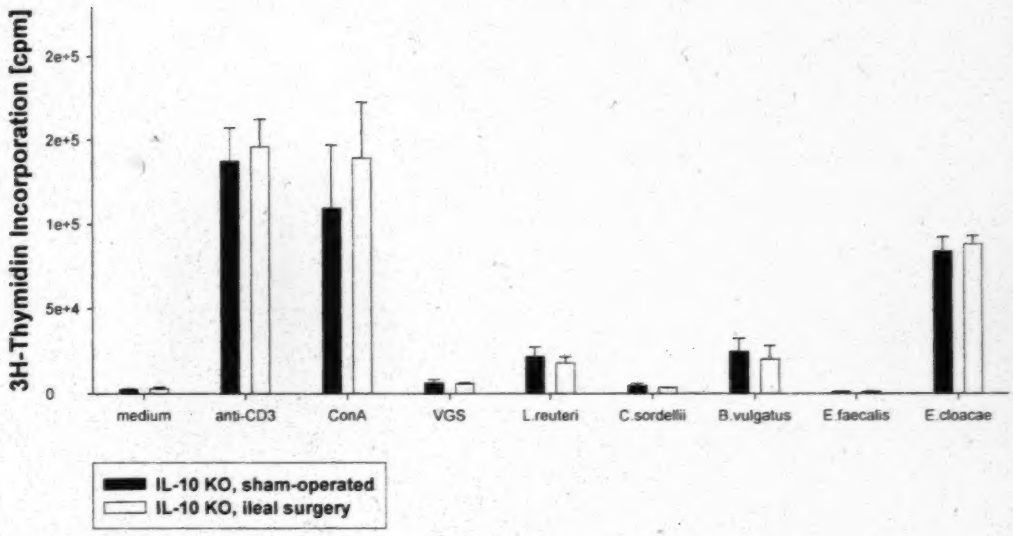

Figure 32: IL-10KO Splenic lymphocyte responsiveness to chemical stimulants and bacterial sonicates as measured by thymidine incorporation (cell proliferation) (n=4 for each group). All comparisons between sham and surgical animals are not significant ($p>0.05$).

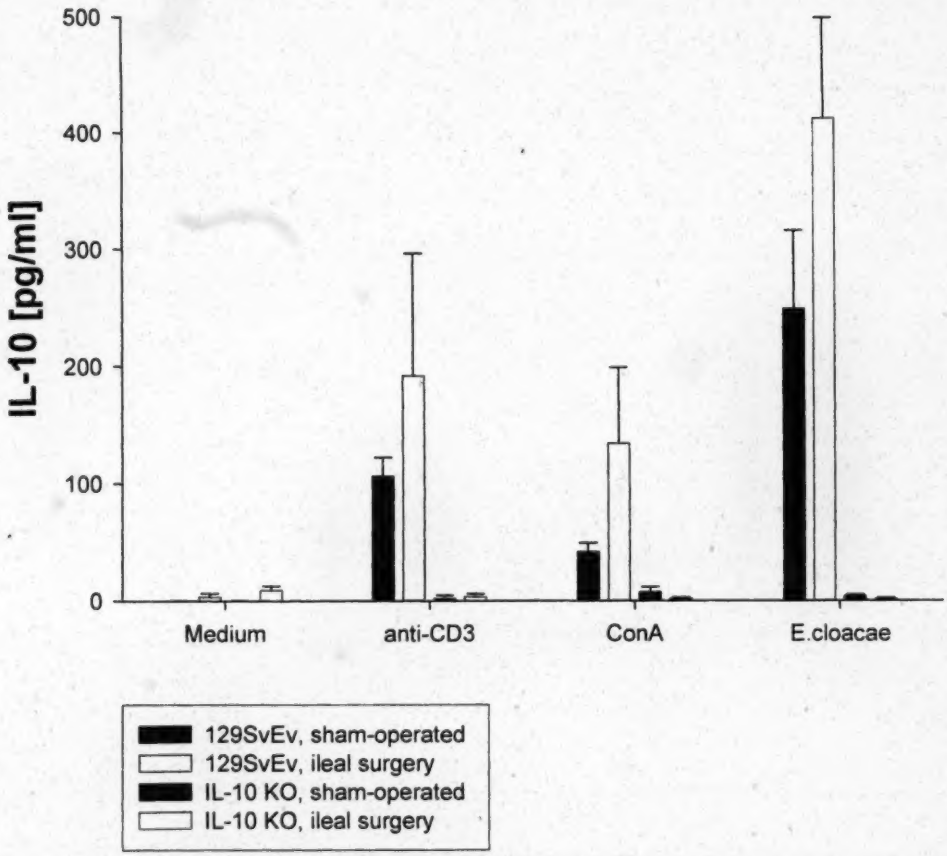

Figure 33: Splenic lymphocyte production of IL-10 in response to anti-CD3, Con A and *E. cloacae*, in WT and IL-10KO animals who underwent sham or surgical procedures (n=4 for each group). All comparisons between sham and surgical animals are not significant ($p>0.05$).

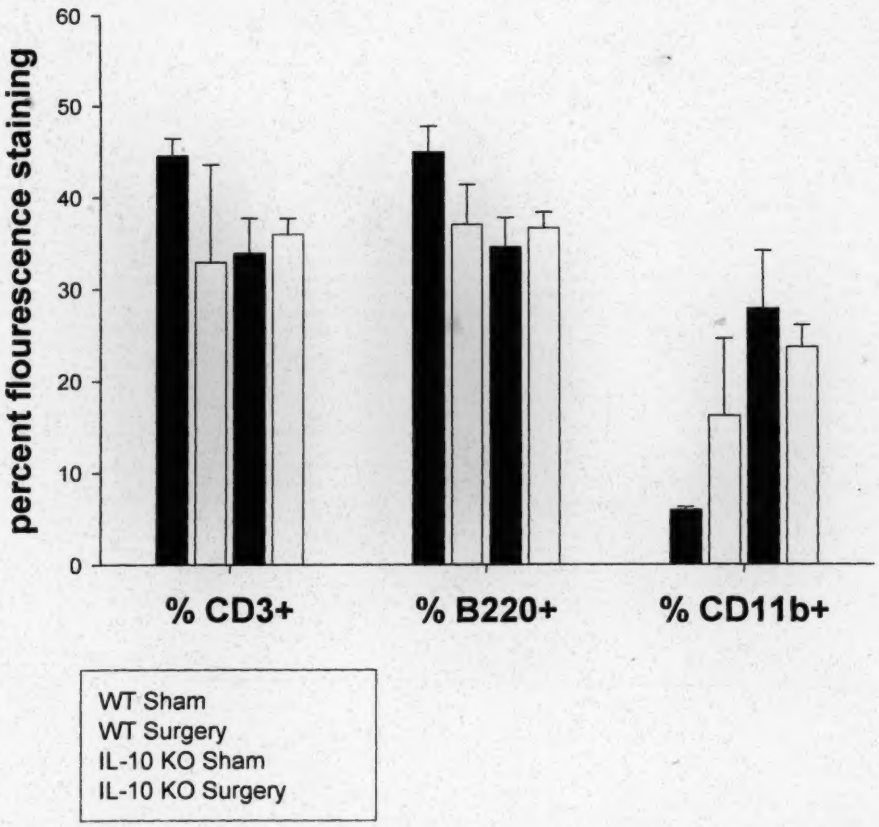

Figure 34: Flow cytometry of day 7 postoperative spleen-cell populations. CD3 is a marker of T-cells, B220 is a marker of B-cells, and CD11b is a marker of macrophages.